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EXTRACELLULAR PROTEASES OF A SPECIES
OF PSEUDOMONAS

BY

Ⓒ JOHN WALTER CHERWONOGRODZKY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Extracellular Proteases of a Species of Pseudomonas submitted by John Walter Cherwonogrodzky in partial fulfilment of the requirements for the degree of Master of Science in Microbiology.

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DATED October 20, 1977.

DEDICATION

The following thesis is dedicated to members of my family.

Always out of sight, never out of mind.

ABSTRACT

An isolate from contaminated steerhides, Pseudomonas 461-3-11, was found to produce 3 extracellular proteases. These enzymes were isolated and purified by $(NH_4)_2SO_4$ fractionation and ion-exchange chromatography (DEAE and CM-cellulose). An increase in specific activity of 28 times that of the crude material was obtained. Except for a minor inactive contaminant for Protease 3, all enzymes yielded active single bands on discontinuous polyacrylamide gels (pH 4.3).

Production of proteolytic activity was found to occur during the growing phase and was not dependent upon cell autolysis. The optimum temperature for the growth of Pseudomonas 461-3-11 was found to be 33-34°C. Protease production was greatly influenced by temperature, being 50 fold greater at 26°C than at 38°C in comparison with growth (OD 600 nm.) being 2 fold greater respectively. In a similar manner, the degree of aeration was found not to delay but to decrease the production of protease. Under identical conditions, trypticase soy broth gave the highest enzyme production, brain-heart infusion broth gave the highest yield of growth, while nutrient broth gave both the least growth and proteolytic activity. Attempts to grow Pseudomonas 461-3-11 in synthetic media were unsuccessful.

The three extracellular enzymes, labelled Proteases 1, 2, and 3 were shown to have molecular weights of 31,000, 33,000, and 59,000 respectively. Only Protease 3 was found to solublize collagen. The pH optima for these enzymes were 9.3, 9.5, and 8.5 respectively, and all three were sensitive to EDTA although Protease 3 was far more resistant than the other two. The significance of the similarities

of Protease 1 and 2, and the differences between these with Protease 3 are discussed at the end of this text.

Research by definition is an analysis of the unknown. If one relies totally on what is known or what has been taught, then that individual will always be self-limiting. Intuition, imagination, and luck may not be very scientific but they are a vital part of research.

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ABBREVIATIONS

A	absorbance
Δ A	a change in absorbance (increase)
BIS	N_1N_1-methylenebisacrylamide
BSA	bovine serum albumin
CM	carboxymethyl
DEAE	diethylaminoethyl
DFP	diisopropylfluorophosphate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(β-aminoethylether) N_1N^1-tetraacetic acid
eu.	enzyme units
IEF	isoelectricfocusing
IgG	immunogammaglobulin
OD	optical density
Δ OD	a change in optical density (increase)
PCMB	p-chloromercuribenzoate
PEG	polyethylene glycol
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TCS	trypticase soy broth
TEMED	$N_1N_1N_1N_1$-tetramethylethylenediamine
TRIS	tris(hydroxymethyl)-aminomethane

INTRODUCTION

Significance of Pseudomonas Species

During the first part of the twentieth century, diphtheria, scarlet fever, tuberculosis and pneumococcal pneumonia were common fears that the general practitioner had to contend with. Then, with the widespread introduction of antibiotics, elaborate surgical procedures and increased accessibility to specialized hospital units, cases of Gram-positive bacterial diseases decreased, only to be offset by an increase of those caused by Gram-negatives (Flick and Cuff, 1976). The role of *Escherichia coli* as a puerile pathogen has become more recognized (Hanson, 1976; Neter, 1976), cases of penicillin resistant gonococci have become more prevalent (Ashford *et al.*, 1976), while an extensive survey has shown the presence of antibiotic multiresistance within 60% of *Klebsiella-Enterobacter*, 90% of the *Proteus mirabilis* and 95% of the *Pseudomonas aeruginosa* clinical isolates (Grunt and Krcmery, 1976). As stated by Youmans *et al.* (1975), "Although man can build a better mouse trap, nature always seems to build a better mouse."

Of particular importance within recent years have been infections to the compromised host by *Pseudomonas* species. The usual consequence of corneal ulceration is the loss of an eye (Ayliffe *et al.*, 1966), mucoid strains are prevalent in cystic fibrosis patients (Hoiby, 1974), and on occasion septicemic epidemics have swept through intensive care units (Cabrera *et al.*, 1975; Phillips *et al.*, 1971). Within one hospital ward, for example, of 172 children suffering from burns, 29 developed lesions, 22 of which were caused by *Pseudomonas aeruginosa*. Death occurred within 24-48 hours after the appearance of the first lesion

(Forkner, 1960).

Although the common reservoir appears to be the patient (Flick and Cuff, 1976), other sources have yielded unsettling statistics. In one instance, 64% of saline solution bottles used to moisten the cornea during an operation were subsequently found to contain pathogenic _Pseudomonas aeruginosa_ (Ayliffe _et al._, 1966); in another, sutures used for open-heart surgery were found to be contaminated (DeHaven, 1969); while in another, disinfectant solutions of phenols or alkyl chlorides were found to harbour these organisms (Bassett _et al._, 1970; Elliott _et al._, 1977).

Despite its clinical significance, at times one might feel that the deluge of medical reports is presenting a picture too one-sided. Just as in man the organism is usually present but harmless until the host is subjected to stress (Taylor, 1974), so it acts on fish (Herbert _et al._, 1971) and livestock (Brigham _et al._, 1974; Mehta _et al._, 1971; Shimizu _et al._, 1974). Several species are pathogenic for plants although the literature is contradictory for their general properties. Sands _et al._ (1970) suggests that as a whole the plant pathogens appear less hardy and can use fewer carbon substrates than most species of _Pseudomonas_. Ballard _et al._ (1970) has stated that perhaps the reverse is more common. As for the everyday bachelor or housewife, they are probably more concerned with the spoilage of raw vegetables (Green _et al._, 1974), meat, poultry, or eggs (Ayres, 1960; McMeekin, 1975), and seafoods (Herbert _et al._, 1971). In the past most of the contamination has been attributed to _Achromobacter_. However, with better taxonomic methods, species of _Pseudomonas_ have been found to be the main agents of spoilage (Brown _et al._, 1959).

Extracellular Enzymes

Tissue infections (Wilson, 1970), plant diseases (Keen *et al.*, 1967), food spoilage - despite the differing effects of *Pseudomonas* contamination, extracellular enzymes have been observed and suggested as contributing factors to bacterial invasiveness. Extracellular enzymes for the most part are simply hydrolases of large macromolecules such as lipids (Stinson and Merrick, 1974), carbohydrates (Coleman and Elliott, 1962), nucleic acids (Coleman and Elliott, 1965), or proteins (Tarrant *et al.*, 1973) which cannot readily be transported into the cell.

Although most works deal with one or a few extracellular enzymes, the reader should not assume that all bacterial cells have such limited productions. Most investigations are selective and, aside from a few exceptions, usually pay little attention to other enzymes which may be present. One such exception, as an example, noted 13 extracellular enzymes of differing substrate specificities within culture filtrates of *Pseudomonas fluorescens* (Winters and Corpe, 1971). Finally, routine conditions not taking into account parameters such as temperature (Sadovski and Levin, 1969), pH (Cheng *et al.*, 1971), or the absence of certain cations (Bissell *et al.*, 1971) might inactivate some enzymes, causing the researcher to assume them absent.

Secretion of Extracellular Enzymes

Being extracellular, by definition the enzymes are released by a means other than cell autolysis. As an active form within the cell would be counter-productive, degrading synthesized components, it is unlikely that they ever exist within the cytoplasm as active forms

(Both et al., 1972). How they are assembled or how they are released pose problems basic to microbiology, and a review of the literature presents three models.

i) Secretory Carriers: Within mammals, trypsin inhibitors have evolved with the enzyme to prevent an autocatalytic chain-reaction of the zymogen (Stroud et al., 1975). Other common sources of similar inhibitors are found within such plants as the potato (Rouleau and Lamy, 1975), chick pea (Belew and Eaker, 1976), and soybean (Finkenstadt and Laskowski, 1965). These inhibitors are proteins which vary in their molecular weights (Bryant et al., 1976) but do share the common trait that they combine covalently with the enzyme. In the example of trypsin and the soybean trypsin inhibitor, a suggested mechanism is that the protease cleaves a sensitive peptide bond within the inhibitor. A covalent bond then forms between the active seryl group of trypsin and the newly formed carboxyl terminal of the inhibitor, inactivating the protease (Finkenstadt and Laskowski, 1965).

An inhibitor to its own extracellular ribonuclease was discovered in Bacillus amyloliquefaciens, and its possible role as a secretory carrier was reviewed (Smeaton and Elliott, 1967). In this context, it was speculated that the ribonuclease was synthesized within the cell but then quickly inactivated by the inhibitor to prevent degradation of components in the cytoplasm. The two would then be secreted, the ribonuclease being reactivated upon the release of the inhibitor. Results have indicated, however, that the latter merely prevents any back-diffusion as a result of any change in the cell's permeability (e.g. by a drop in temperature). Further research has noted that the binding is essentially irreversible, masking the active site of the ribonuclease by hydrophobic binding (Hartley, 1970).

ii) Secretory Vesicles: As mammalian systems have revealed specific structures such as the Golgi apparatus for the transport of macromolecules out of the cell (Jamieson and Palade, 1966), so bacteria were once thought to possess such secretory vesicles (Sargent et al., 1968). However, in the study of penicillinase secretion by Bacillus licheniformis, vesicles were present in cells not producing the extracellular enzyme (Ghosh et al., 1968). Also, artifacts resulting from the plasmolysis of Bacillus megaterium cells (Weibull, 1965) are similar in appearance, while the lack of correspondence between freeze-etches and thin-section data (Ghosh et al., 1969) make the significance of these vesicles unclear.

iii) Secretion Through The Cytoplasmic Membrane: Perhaps the most widely accepted model follows that of eukaryotes (Redman, 1967) in which secretion of extracellular enzymes or zymogens occur at the cytoplasmic membrane (Both et al., 1972). In the examples of penicillinase by Bacillus licheniformis, a lipophilic proenzyme becomes associated with the bacterial membrane (Bettinger and Lampen, 1971), and then by the action of a protease (Aiyappa and Lampen, 1977) it is released into the media as a hydrophilic form (Bettinger and Lampen, 1975).

As for the translational site of these enzymes, several investigators believe this to be membrane-associated also (May and Elliott, 1968; Sargent and Lampen, 1970). Within species of Bacillus, most of the cell's ribosomes (Dijk-Salkinoja et al., 1970) and about 25% of its RNA (Yudkin and Davis, 1965) have been demonstrated as membrane-bound. A common trait for the synthesis of extracellular enzymes is that they are usually more sensitive to inhibitors than are most proteins of the cell (Glew and Heath, 1971). This appears to be a property of membrane association (Stinson and Merrick, 1974) although intracellular enzymes such as β-galactosidase or galactokinase (Paigen, 1963; Paigen and Williams, 1970) also exhibit this property. Finally, polyribosomes bearing nascent polypeptides of alkaline phosphatase have been localized at the cytoplasmic membrane of Escherichia coli (Cancedda and Schlesinger, 1974).

Proteases

Of the various extracellular hydrolases, perhaps the proteases are the most interesting because of their wide range of functions:

i) Nutrition: Within Streptococcus lactis (Thomas et al., 1974), the primary role of a proteinase bound to its cell surface was found to be digestion of external proteins. To most people, the stomach and pancreatic enzymes are common knowledge. Although each has its own specificity, combined they can degrade most polypeptides. Trypsin hydrolyzes at the carbonyl group of basic amino acids (Casey and Lang, 1976), chymotrypsin at hydrophobic groups (Lehninger, 1975), elastase at small neutral residues (Largman et al., 1976), while pepsin cleaves at the imino function of hydrophobic or acidic amino acids (Fruton, 1975). A generality that may give further insight into the nature of a protease has been suggested by Neurath (1975). If the enzyme or collection of enzymes have extremely broad or overlapping areas of specificity, then their function is probably the hydrolysis of polypeptides for nutrition. If, however, cleavage is specific, such as only at flexible regions or exposed loops of the substrate (Naslin et al., 1973; Johnson and Smillie, 1974), then their function may be one of activation rather than degradation.

ii) Activation: Many enzymes, hormones, or physiologically active proteins such as complement or the blood-clotting factors are synthesized as inactive precursors then converted to active forms by selective cleavage of peptide bonds (Neurath and Walsh, 1976). One example of such limited proteolysis for activation are the pancreatic enzymes mentioned above (Kassall and Kay, 1973). In a similar manner, activation of a mammalian macrophage collagenase is by a neutral protease secreted by the same cell (Horwitz *et al.*, 1976).

iii) Development: The activation of cocoonase after the maturation of insect larvae (Felsted *et al.*, 1973), cleavage of viral proteins for sequential assembly (Laemmli, 1970), and activation of chitin synthetase for separation of yeast mother and daughter cells (Cabib and Farkas, 1971) are different examples where proteases play a key role in development. Within bacterial cells, spores of *Bacillus* species carry out no detectable endogenous metabolism or macromolecular synthesis (Sakakibara *et al.*, 1965). Upon germination, however, proteolytic enzymes degrade specific proteins within the interior of the spore to generate amino acids for protein synthesis (Setlow and Primus, 1975; Setlow, 1976). Within the vegetative cell, proteases as yet do not appear to be directly significant for cell wall synthesis. For growth, degradation is essential, cleaving existing bonds to allow insertion of new segments of completed precursors (Shockman, 1965). For this hydrolysis, however, in the example of *Streptococcus faecalis*, only a native muramidase appears to be present (Boothby *et al.*, 1973; Rosenthal *et al.*, 1975; Cleveland *et al.*, 1976).

iv) Microbial Antagonism: Aside from the release of penicillinase from *Bacillus licheniformis* membranes (Aiyappa and Lampen, 1977), very little attention has been paid to proteases and their role in microbial antagonism. A strain of *Pseudomonas fluorescens* has been noted to produce a mixture of exoenzymes which degrade cell envelopes of Gram-negative bacteria (Whiteside and Corpe, 1969; Winters and Corpe, 1971) while a strain of *Pseudomonas aeruginosa* produces an endopeptidase that cleaves the pentaglycine cross-bridges of *Staphylococcus aureus* cell wall peptidoglycan (Lache *et al.*, 1969). Possibly as conditions go from an agar plate back to the field, observations of this nature will become more evident.

Classification of Proteases

Although the function of a protease states why it is there, characterization resolves how it acts. For the latter, classification can take several directions. The effects of inhibitors (Walsh, 1975; Schnebli, 1975) homologous sequences and similar chain folding (Neurath *et al.*, 1967; Titani *et al.*, 1972), action on specific substrates such as by collagenase (Carrick and Berk, 1975) or keratinase (Nickerson and Durand, 1963), and the nature of activity [whether the enzyme acts as an endopeptidase (Burstein *et al.*, 1974) or exopeptidase (Schmid and Herriott, 1974)] are all feasible but the most widely used is by the mechanism of action (Morihara, 1974; Walsh, 1975). Rather than a single manner by which a protease can hydrolyze a polypeptide, basically four have been resolved depending on their mechanism and constituent groups at the active site. Rather than go into any length over the differences between or within the four major sets, brief outlines of their general characteristics have been presented below.

i) Acid Proteases: Although pepsin is the model for this group (Fruton, 1970), within the microbial world they can be found as extracellular enzymes of yeasts or moulds (Matsubara and Feder, 1971). Having pH optima between 2-4, hence their designation (Morihara, 1974), the key amino acid within their active site appears to be aspartic acid (Sodek and Hofman, 1970; Kovaleva et al., 1972). For the degradation of their substrates, Fruton (1970) has speculated on the concerted action of two acidic residues within the enzyme on the substrate's peptide bond. One acidic group's carboxyl acts as a proton donor to the substrate's carbonyl; the other acts as a nucleophile, forming an unstable anhydride with the latter which rapidly undergoes hydrolysis at the peptide bond. This mechanism is further supported by the resistance of the proteinase to inhibitors such as DFP, EDTA, or PCMB, but sensitivity to diazoacetyl-DL-norleucine methyl ester which in the presence of cupric ions binds to aspartic acid (Sodek and Hofman, 1968; Mizobe et al., 1973).

ii) Sulphydryl Proteases: Papain from papaya fruits, cathepsins of animal tissues, streptococcal proteinases, and clostridio-peptidase B are all examples of the set that has a free sulphydryl group essential for activity (Drenth et al., 1971). Activation is effected by reduction of disulphide bonds by agents such as cyanide, cysteine, or glutathione; while inactivation occurs if the sulphydryl is alkylated by compounds such as iodoacetate, methylamine, or methylguanidine (Mitchell and Harrington, 1971). Despite controversy over its mode of hydrolysis on polypeptides, essentially it is believed to use a two-step process. In papain, the reactive cysteine has been shown to form an acyl-thioenzyme complex with the substrate which is then deacylated by the carboxyl ion of aspartic acid, acting as a base (Glazer and Smith, 1971). Whether a proximal histidine either within papain or a streptococcal proteinase plays a major role in this mechanism or not has yet to be determined (Gerwin et al., 1966; Clark and Lowe, 1976).

iii) Serine Proteases: Found within bacteria, moulds, or yeasts (Pacaud and Uriel, 1971; Turkova et al, 1971; Hayashi and Hata, 1972), these enzymes share similarities in mechanism with the pancreatic enzymes (Morihara and Tsuzuki, 1968). Trypsin, chymotrypsin, and elastase all have within their catalytic sites a serine, histidine, and aspartic acid residue (Blow et al., 1969). Compounds such as DFP that react with the serine by alkylphosphorylation (Morgan et al., 1972) inactivate these enzymes, hence their designation. Initially the hydrophobic environment within the reactive pocket allows the transfer of a neutral aspartic acid's proton to a neutral histidine at pK's of 6.8 (Koeppe and Stroud, 1976). With the serine these then form a tetrahedral intermediate with the substrate (Stroud et al., 1975) and hydrolysis proceeds by the shuttle of protons. Silver ions that bind between the aspartic and histidine residues, therefore, can also be strong inhibitors (Chambers et al., 1974). Differences in pH optima (Morihara, 1974) or specificities may be accounted for by the sizes or charges of amino acid side groups within this pocket (Krieger et al., 1974; Shotton and Watson, 1970).

iv) Metal-Chelator-Sensitive Proteases: Although metal ions such as calcium or zinc have been known to stabilize several enzymes (Bissell et al., 1971; Trotman and Greenwood, 1971; Dahlquist et al., 1976), within this class of proteases the metal is also essential for activity.

They are, therefore, sensitive to chelating agents such as EDTA or o-phenanthroline but seldom to other reagents such as DFP, PCMB, or soy-bean inhibitors (Morihara, 1974).

Despite an enzyme's sensitivity to chelating agents, one should be cautious about classifying it within this category simply by noting inhibition by EDTA, for example. For thermolysin, calcium ions are used for stability while zinc is at the active site (Dahlquist *et al.*, 1976). It is possible, therefore, to have an enzyme that does not have a metal at its active site but requires such a component for its stability. Enzyme 2 of *Pseudomonas aeruginosa* (Morihara, 1964) is sensitive to iodoacetate suggesting a sulphydryl protease, while all three enzymes of *Serratia* NRC 1004 are sensitive to DFP (Nunokawa and McDonald, 1968) suggesting a serine protease. These are also sensitive to EDTA and so one cannot adequately classify them without further clarification. Finally, Morihara and Tsuzuki (1964) have observed that for the peptide peptidohydrolase of *Pseudomonas aeruginosa*, EDTA inhibits the enzyme simply by masking the active site.

For substrate hydrolysis, using carboxypeptidase A as a model, there is still much controversy over the mechanism (Hartsuck and Lipscomb, 1971; Breslow *et al.*, 1975). A compromise has been suggested in which both the arginine and tyrosine residues within the active site restrain the substrate's free carboxyl group while zinc and glutamate actively hydrolyze the peptide (Breslow and Wernick, 1976). Other traits of these enzymes are that alkaline proteases are more common than neutral proteases (McConn *et al.*, 1964) and that the latter are more sensitive to EDTA (Morihara, 1974). Also, Protease I of *Myxobacter* AL-1 has the unusual property of being able to lyse cell walls of some bacteria (Ensign and Wolfe, 1966; Jackson and Wolfe, 1968).

Extracellular Proteases of Gram-negative Bacteria

Proteases can be found in everything from insects (Garcia and Garcia, 1977) to carnivorous plants (Clancy and Coffey, 1977); yet, for the Gram-negative bacteria, works on extracellular proteases are sparse. A few researchers have generalized that they are produced by only certain strains of pseudomonads or vibrios (Pollock, 1963; Costerton et al., 1974). Although there are significant differences between Gram-positive and Gram-negative cell wall peptidoglycan (Braun and Sieglin, 1970; Heilmann, 1972), Stinson and Merrick (1974) have suggested that the restriction of protease production might instead be a reflection of the outer membrane or envelope of Gram-negatives. The "leaky" nature of some species (Lopes et al., 1972), release of periplasmic enzymes by high molarity solutions (Cheng et al., 1970; Day and Ingram, 1971; Ingram et al., 1973) or by the formation of spheroplasts (Cheng et al., 1971; Bhatti et al., 1976) give added support to this belief. However, upon scanning the sections dealing with Gram-negatives within Bergey's manual (Buchanan and Gibbons, 1975), one will frequently find statements that several organisms "...liquefy gelatin ...liquefy gelatin over several weeks...[or cause] liquefaction in less than half the time of controls." Therefore, the statement that extracellular proteases of Gram-negatives belong only to certain strains of pseudomonads or vibrios may be misleading, based on limited investigations. The following are brief summaries of the reported species within Gram-negative bacteria:

i) Enterobacteriaceae: As early as 1949, Simmonds and Fruton reported that mutant strains of *Escherichia coli* which needed certain amino acids for growth could utilize synthetic peptides as long as the peptides contained the essential amino acids. In more recent times an aminoendopeptidase has been located within the periplasmic space of Gram-negatives (Murgier *et al.*, 1976). With *Escherichia coli* K-10, studies of this hydrolase seems to yield more questions than answers. For aminopeptidase activity (hydrolysis of L-alanine p-nitroanilide) its pH optimum is 7.0, while for endopeptidase activity (hydrolysis of casein) the optimum is 7.5 (Lazdunski *et al.*, 1975c). Regulation is not by limiting nitrogen or carbon sources but instead by the extracellular level of inorganic phosphate (Lazdunski *et al.*, 1975b). Osmotic shock or treatment with EDTA and lysozyme are effective for the release of alkaline phosphatase, another periplasmic enzyme, but are only partially successful in releasing the protease (Lazdunski *et al.*, 1975a). Finally, although most extracellular proteases of bacteria are metalloenzymes, this one is a sulphydryl protease, inactivated by N-ethylmaleimide but not by EDTA (Lazdunski *et al.*, 1975c).

A strain of *Citrobacter freundii* produces an extracellular protease with a molecular weight of 45,000. The latter is insensitive to DFP or PCMB but strongly inhibited by metal chelators. One mole of zinc has been found to be incorporated within one mole of enzyme (Nakajima *et al.*, 1974).

Serratia marcescens strain NCIB 10351 synthesizes a zinc metalloprotease of a molecular weight of 30,000. It hydrolyzes casein or tendon collagen at an optimum pH of 9 yet cleaves synthetic substrates optimally at pH 5. The hexapeptide, carbobenzoxy-gly-pro-gly-gly-pro-ala, is cleaved between the two adjacent glycine residues (McQuade and Crewther, 1969) which is suggestive for collagenase activity (Schoellman and Fisher, 1966). The hydrolase does not appear if the strain is subcultured in synthetic media but is induced by the addition of casein (Castaneda-Agullo, 1955) and repressed by the presence of carbohydrates such as glucose (Ryden and Hofsten, 1968).

The extracellular protease of a Serratia sp. pathogenic for silkworms has been purified and found to be sensitive to EDTA near neutrality but not to EDTA above pH 8.0, iodoacetate, PCMB, or DFP (Miyata et al., 1971). Aside from casein which is readily degraded, the enzyme can hydrolyze denatured lysozyme, insulin, or IgG but is severely restricted by native forms of these substrates (Miyata et al., 1970b). Zinc is the prosthetic group (Miyata et al., 1971) which when removed and replaced by other metals will give different stabilities or proteolytic rates (higher for cobalt, lower for magnesium for both properties). The enzyme has no detectable sulphur containing amino acids such as cysteine or methionine (Miyata et al., 1970a), a property once thought to be a universal trait of extracellular enzymes (Pollock, 1963). However, as several extracellular proteases do have these residues (Prescott et al., 1971; Carrick and Berk, 1975; Lazdunski et al., 1975c), the low abundance rather than the complete absence of sulphur containing amino acids appears to be more accurate.

Other work on a Serratia related species has been with the obligate psychrophilic strain NRC 1004 which produces three proteases. The three are similar in that DFP or EDTA inactivate them while o-phenanthroline, PCMB or iodoacetate do not (Nunokawa and McDonald, 1968). The sensitivity of these enzymes to DFP suggests that they are serine proteases. All three enzymes exhibit an optimum pH of 9.8-10.8 and an optimum temperature of 40°C in contrast with the protease of Serratia marcescens which has an optimum pH and temperature of 9.0 and 45°C respectively (McDonald and Chambers, 1963).

In 1916, Jones reported the repression of proteolytic activity of Proteus vulgaris by the presence of carbohydrates. Aside from a few papers (Walker, 1927; Gorini, 1950), it wasn't until several years later that the presence of two enzymes with pH optima between 7.2-7.6 and one more heat stable than the other (Bensusan et al., 1954) was reported.

Proteus mirabilis, has but one protease that has a high tyrosine and aspartic acid content and is irreversibly inactivated by EDTA. An unusual property of this enzyme is that although it cleaves the cysteic acid-glycine bond 7-8 of oxidized insulin B-chain, the identical bond at 19-20 is not hydrolyzed (Hampson et al., 1963).

ii) Vibrionaceae: In Vibrio parahaemolyticus, although attention has focused primarily on its thermostable haemolysin (Sakurai et al., 1975; Honda et al., 1976), an extracellular proteinase is also produced. Sensitive to EDTA and requiring de novo protein synthesis, the enzyme is repressed by metabolizable carbon such as glucose, glycerol, or casamino acids but stimulated either by gelatin or its partial hydrolysate (Tanaka and Iuchi, 1971).

Studies on Aeromonas proteolytica revealed two extracellular enzymes, an aminopeptidase and an endopeptidase (Prescott and Wilkes, 1966). The former has been more extensively studied and has a molecular weight approximately 29,000, and is unusually high in acidic amino acids but low in basic residues, and has 2 atoms of zinc as its prosthetic groups (Prescott et al., 1971). The highest yield within a culture occurs when asparagine or sodium nitrate with glycerol are added to the medium. A partial hydrolysate of casein also greatly stimulates its production (Litchfield and Prescott, 1969b). For the latter enzyme, although a partial hydrolysate of casein also increases its yield, asparagine or sodium nitrate with glycerol represses rather than induces its production (Litchfield and Prescott, 1969a). It should be noted that in a growing culture, a peak of aminopeptidase production occurs first and then only after several hours does the endopeptidase peak appear (Prescott and Wilkes, 1966).

iii) Pseudomonadaceae: In 1963, Mandl stated "all evidence points to the conclusion that collagenolytic activity is uniquely associated with Clostridium histolyticum, Cl. perfringens, and possibly Cl. captivole," believing that results to the contrary came from faulty preparations of the substrate. Waldvogel and Swartz (1969) reported negative results for collagenase production by Pseudomonas aeruginosa in spite of the fact that proteases were produced that were capable of hydrolyzing carbobenzoxy-gly-pro-gly-gly-pro-ala. Brown et al. (1974) reported similar findings while in 1975, Gray and Kreger expressed their opinion that in cases of Pseudomonas infections of the eye, corneal damage was due to the release of polymorphonuclear lysosomal enzymes rather than collagenases from the bacteria.

Pseudomonas aeruginosa and its collagenases have been of considerable interest due to corneal infections stemming from either the improper antisepsis associated with the use of contact lenses (World Contact Lens Report, 1959) or in the production of cosmetics (Marzulli et al., 1972). Although the hydrolysis of carbobenzoxy-gly-pro-gly-gly-pro-ala might not be sufficient to label a protease a collagenase

(Schoellman and Fisher, 1966), reports of Pseudomonas aeruginosa proteases degrading native collagen are present (Fisher and Allen, 1958; Horn et al., 1973; Carrick and Berk, 1975). Differences in results might be due either to long lag periods for the appearance of the collagenase (Adamcic and Clark, 1970), the variation in cross-linkages of the substrate (Giffee, 1971), or the variation between observed strains. Brief reviews rather than comparisons are therefore given below.

Five proteases labelled A to E have been isolated from a strain of Pseudomonas aeruginosa pathogenic for the larvae of Galleria mellonella. All five are sensitive to EDTA, their pH optima range from 7.5 to 9.0, their molecular weights are 13,000 to 70,000, all but A are collagenolytic while all but D are elastolytic (Kucera and Lysenko, 1968).

Kreger and Griffin (1974) purified 3 proteases that have a molecular weight of approximately 20,000, are inactivated by 15 minutes at 80°C, have isoelectric points of 4.6, 5.8, and 8.8, and are not produced if the culture is grown in 4.7% ammonium sulphate.

Through ethanol fractionation, ion-exchange chromatography and gel filtration, Carrick and Berk (1975) have isolated a single collagenase after a 1575 fold purification and 24% recovery. Ferrous chloride, cysteine, EDTA, or ammonium sulphate inactivated the preparation while after treatment with metal-chelators, cobalt or zinc ions reactivated proteolytic activity. The pH optimum was 7.5 although activity was greater in TRIS than in phosphate buffers of the same ionic strength or pH. An unusual characteristic of the collagenase is that by gel exclusion chromatography its molecular weight was determined as 17,500, while on SDS polyacrylamide gels it was 34,000. The authors attributed this discrepancy to dimer polymerization above pH 8.0.

As most reported collagenases have been found to be metalloproteins, attempts have been made to stop corneal degradation in cases of Pseudomonas infections of the eye by applying solutions of EDTA. Results have been contradictory, ranging from claims of considerable improvement (Brown et al., 1969) to no apparent influence (Bohigian et al., 1974). Wilson (1970) has stated that possibly calcium found in tears or within the cornea might be the source of the discrepancy, while Liu and Hsieh (1969) have speculated that the proteases under investigation might slow rather than increase corneal infections by degrading exotoxins produced by the microbe.

Aside from collagenases, some strains of Pseudomonas aeruginosa produce other proteases, notably elastase. Rather than the hydrolysis of peptide bonds, elastolytic activity specifically refers to unravelling and solublization of the substrate (Hall and Czerkawski, 1961a; Hall and Czerkawski, 1961b) although most elastases have both properties (Morihara and Tsuzuki, 1967). Characteristics of these enzymes may vary with the strain (Suss et al., 1969; Morihara and Tsuzuki, 1966) but studies have shown that most exhibit a molecular weight of approximately 40,000, and sensitivity to EDTA, phenanthroline, molar sodium chloride, or metals such as mercury, nickel, and zinc. The pH optimum is difficult to determine as it varies with the ionic strength and type of buffer, being 8.0 in 0.03 M TRIS, 7.2 in 0.10 M TRIS, 7.5 in 0.03 M phosphate, or 7.0 in 0.03 M carbonate (Morihara et al., 1965).

Several reports indicate that other proteinases are also secreted with the elastase (Morihara, 1964; Morihara and Tsuzuki, 1966; Morihara and Tsuzuki, 1967). One of the accompanying proteases has been characterized to have a molecular weight of 48,400 (Inoue et al., 1963), requires calcium for stability (Morihara et al., 1973), has an optimum pH between 7-9, and has an optimum temperature of 60°C. It is also inactivated by EDTA, o-phenanthroline, or oxidizing agents such as potassium permanganate (Morihara, 1963). As the effects of chelators are reversed by simply dialyzing against distilled water, it is believed that EDTA merely masks the active centre without removing the constituent metal (Morihara and Tsuzuki, 1964). Another endopeptidase, although not extensively studied due to its low yield, is unusual in that it appears to be common to several strains of Pseudomonas aeruginosa and is insensitive to EDTA or phenanthroline (Morihara, 1964).

Within the previous section dealing with protease function and microbial antagonism, it was stated that a strain of Pseudomonas aeruginosa produces an enzyme lytic for species of Staphylococcus (Zyskind et al., 1965; Lache et al., 1969). The enzyme has been analyzed to the extent that it has a sharp pH optimum of 8.0, is inhibited by 0.07 M TRIS, and is inactivated by EDTA, mercury, or copper ions (Burke and Pattee, 1967).

Aside from Pseudomonas aeruginosa, other species have had their proteases characterized. During pathogenesis, Pseudomonas lachrymans, the casual agent of cucumber angular leaf spot, produces a proteinase that can hydrolyze casein, haemoglobin, or cucumber leaf proteins but not collagen, elastin, or gelatin (Keen et al., 1967). Inhibition of this enzyme is by zinc or cobalt ions while EDTA, DFP, PCMB, KCN, or iodoacetate have no effect. Also, autoclaving for 10 minutes within a buffer of pH 11.0 (its optimum is pH 8.0) will activate the enzyme. As for its induction, addition of proteins to the growth media have little influence, while the production of the proteolytic enzyme increases if either sucrose or glutamic acid levels are increased to 0.2%. Above these concentrations, enzyme production again decreases (Keen and Williams, 1967).

A serine protease from Pseudomonas maltophilia has been purified by ammonium sulphate precipitation, Sephadex G-75, and Bio-rex gel chromatography. Although the recovery was 37% and the specific activity merely doubled, polyacrylamide gels have revealed only a single band, suggesting an absence of cell lysis and that the enzyme constitutes a major portion of the extracellular proteins. The pH optimum for this enzyme was found to be 10.0, and although it is insensitive to iodoacetate, both EDTA and DFP inactivate it (Boethling, 1975).

Pseudomonas fragi produces a chelator-sensitive protease that is activated by zinc and stablized by calcium. It has an optimum pH between 6.5-8.0, an optimum temperature of 40°C, and a molecular weight of 40,000-50,000 (Porzio and Pearson, 1975). The enzyme is extremely heat-labile, losing 50% of its acitivity upon incubation at 35°C for 5 minutes. Also, growth within a Koser's citrate medium having ammonium as the nitrogen source will inhibit the microbe's production of the protease while addition of several amino acids with a few dipeptides will cause its production to be unimpaired (Tarrant et al., 1973).

Other Pseudomonas extracellular proteases that have been characterized to some extent are those belonging to psychrophiles. A psychrophilic halophile has been isolated which requires high salt levels not only for growth but also for the production of its two proteases. If the temperature rises above 5°C, it has been found that the relative proportions of these two enzymes will vary, shifting to an increase of one over the other (Kato *et al.*, 1972). A psychrophilic species of *Pseudomonas* isolated from the arctic was found to produce a protease which hydrolyzed gelatin, casein, or β-lactoglobulin but not bovine serum albumin. It was inhibited by EDTA and lost 90% of its activity after being left at 40°C for 25 minutes (McDonald *et al.*, 1963).

Although McDonald *et al.* (1963) have suggested that psychrophilic bacteria might produce heat-labile enzymes, studies with *Pseudomonas spp*. responsible for milk spoilage suggest the contrary to be true. Within 70-90% of all raw milk samples, psychrophiles are present which produce heat resistant proteases (Bengtsson *et al.*, 1973; Adams *et al.*, 1975). Despite sterilization (Mayerhofer *et al.*, 1971), the proteases continue to degrade milk for several days, causing clearing, gelation, or bad flavours (White and Marshall, 1973). The extracellular enzyme of *Pseudomonas fluorescens* P26, as an example, has zinc as its prosthetic metal and is stabilized by calcium. Although when in milk it still retains 10% of its activity after 90 seconds at 149°C (Barach *et al.*, 1975), it is sensitive to metal chelators (Barach *et al.*, 1976) and will lose 90% of its activity within 10 minutes at 55°C if purified and left in buffer (Barach *et al.*, 1975).

In 1965, Giffee *et al.* reported three *Pseudomonas* strains isolated from contaminated steerhides that contributed to the dispersal and solublization of the substrate collagen. The following investigation was undertaken to purify and characterize the proteases of one of these strains, *Pseudomonas* 461-3-11, and to study factors in the stimulation of its activity and production.

MATERIALS AND METHODS

I Materials:

Chemicals, reagents, or solvents used were of reagent grade and obtained from commercial sources. The azocoll substrate (50-100 mesh, lot number 510110) was purchased from Calbiochem. Sephadex dextran beads and Blue Dextran 2000 were made by Pharmacia Fine Chemicals. CM-cellulose cation exchanger (medium mesh, 0.60 meq./gm. capacity, lot number 87B-3110) and DEAE-cellulose anion exchanger (medium mesh, 0.88 meq./gm. capacity, lot number 30C-2370) were purchased from Sigma Chemical Company. Ampholytes were purchased from LKB Produkter.

II Organism and Culture Conditions:

1) Stock Culture: the organism used throughout the investigation has been designated as *Pseudomonas* 461-3-11. It was originally isolated from curing steerhides and was classified by standard taxonomic tests as belonging to the genus *Pseudomonas* but not assignable to any existing species (J. N. Campbell and M. E. Rhodes, personal communications, 1961). Stock cultures were maintained by the weekly transfer to plates of 3.0% gelatin and 1.6% agar in 0.01 M NaH_2PO_4-NaOH buffer pH 7.2 at room temperature. For the production of protease, 3.0% TCS was inoculated with a loopful of growth from the gelatin-agar plate and subcultured at least twice at 30°C at 300 rpm. for 24 hours.

2) Culture Media: The media was prepared according to the manufacturer's specification. Bacto Nutrient Broth and Bacto Brain-Heart Infusion were both produced by Difco Laboratories, Trypticase Soy Broth was purchased from Baltimore Biological Laboratories.

3) The Microfermentor: For large volumes, 5 litre cultures were grown in a Microferm Fermentor, Model 214 (New Brunswick Scientific Co.) with a forced aeration of 5 litres/min., an agitation of 300 rpm. for the mixing paddles, and a temperature of 26°C. A 1.0% inoculum was used and during incubation of the cultures, Antifoam Spray A (Dow Corning Corp.) was used as necessary.

III Cell Counts:

Total cell number was estimated using a Petroff-Hausser counting chamber. Viable cell numbers were estimated by plate counts using 0.1 ml. volumes on 3.0% TCS agar and 24 hours incubation at 30°C.

IV Cell Disruption:

Cell breakage was done with a sonic oscillator Model Biosonik III (Bronwill Scientific Co.). The maximal power output was 300 watts and a 3/8 inch probe was used at 20,000 cps. Samples were contained in a 10 ml. beaker and were sonicated for 15 second intervals. Between these times the beaker and the probe were cooled on ice.

V Absorbance and pH:

Absorbance was measured with a Beckman-Gilford Spectrophotometer. The monochromator was a Beckman Model 2400 while all other components were made by Gilford Instruments Laboratories. For a scan of absorbance a Unicam SP 8000 Ultraviolet Recording Spectrophotometer with an SP 8005 Programme Controller (both by Pye Unicam Ltd.) was used.

pH was determined using a Metrohm pH-Meter, Model E300B.

VI Purification Techniques:

1) Gel Exclusion Chromatography: 2.5 cm. X 38 cm. columns of G-25, G-50, and G-100 (Pharmacia Fine Chemicals) were equilibrated and eluted with 0.01 M TRIS-HCl buffer pH 7.5, at 4°C. A pressure head of 10 cm., and the elution flow of 0.5 ml./min. were approximated for all runs. The void volume was determined using Blue Dextran 2000, the total volume with $FeCl_3$, both at a concentration of 1 mg./ml of buffer.

2) Anion Exchange Chromatography: As both the buffers and column dimensions varied considerably in accordance with the design of different experiments, specifics are noted in Results and Discussion. The flow rate was 0.8 ml./min., the temperature of operation was 4°C. Elution was done using a 0 to 1.0 M NaCl gradient within the specific buffer having a total volume of 400 ml.

3) Cation Exchange Chromatography: Various combinations of buffers and column dimensions were used and are specified in the Results and Discussion. The flow rate was 0.8 ml./min., the temperature of operation was 4°C, and elution was as before except that a 0 to 0.3 M NaCl gradient in the specific buffer was used.

4) Ultrafiltration: For large volumes of several litres, the method of choice was a Model TC1C filtration apparatus (Amicon Corp.) with a 15 cm. diameter PM 10 membrane. The pressure used was 40 psi. compressed air with 20 psi. nitrogen for a total of 60 psi. The filtration was conducted over 4 days at 4°C.

For smaller volumes of a few hundred ml., Model 400 with a 7.5 cm. diameter PM 10 membrane at 10 psi. was used. Concentration was usually continued until a final volume of 10-20 ml. was obtained.

5) Ammonium Sulphate Fractionation: Ammonium sulphate was added

to yield a 36% saturation at 4°C (200 gm./litre). The solution was left for 8 hours at 4°C, centrifuged at 27,000 X g for 15 min., then the pellet was discarded. Another 200 grams of solid ammonium sulphate was added as before (65% saturation at 4°C), the solution was left for 8 hours, then centrifuged at 27,000 X g for 15 min. The pellet was saved, suspended in 10-15 ml. of distilled water, then dialyzed against normally 2 litres of buffer (changed once).

6) Isoelectric Focusing: An IEF apparatus, LKB Model 8100-1 (LKB Produkter) with a 110 ml. capacity column was used and cooled with running tap water (16°C). The power source was Model HV 1000 CVR (Savant Instruments, Inc.). The following solutions were used:

i) Lower Dense Electrode Solution (pH 11.7)

Sucrose	16 gm.
Distilled water	10 ml.
1 M NaOH	6 ml.

ii) Mixing Chamber Solution

Ampholine carrier ampholyte (pH range 3.5-10.0)	1.35 ml.
Sucrose	27 gm.
Sample (salt free, 0.5 mg. protein/ml.)	20 ml.
Distilled water	15.65 ml.

iii) Reservoir Chamber Solution

Ampholine carrier ampholyte (pH range 3.5-10.0)	1.35 ml.
Sucrose	2.7 gm.
Sample (salt free, 0.5 mg.	

protein/ml.)	40 ml.
Distilled water	11.65 ml.

iv) Upper Light Electrode Solution (pH 2.5)

15% (v/v) H_3PO_4	1.5 ml.
Distilled water	8.5 ml.

The anode (+) was connected to the top while the cathode (-) was connected to the bottom of the column. The initial current was 16 mA., 300 volts, and 5 watts. After 36 hours the values were 1 mA., 1000 volts, and 1 watt.

VII Proteolytic Assays: (Assays are designated by their substrates)

1) Azocoll Assay: The method of Moore (1969) was used with the following modifications. Five ml. of 0.01 M TRIS-HCl buffer pH 7.5 and 25 mg. of azocoll were added to each 50 ml. Erlenmeyer flask. These were agitated at 200 rpm. at 37°C for 15 minutes. After preincubation, at 1 minute intervals, 0.01 to 0.05 ml. of samples to be assayed for proteolytic activity were added. After an additional 15 min. incubation, the flasks were removed and the mixture filtered through a Whatman No. 2 filter paper under suction. The A 580 nm. of the filtrate was then measured. One unit of enzyme is defined as that amount which causes an increase in absorbance at 580 nm. of 1.0.

2) Haemoglobin Assay: The substrate was prepared by the method of Anson (1938) with modifications noted by Giffee *et al.* (1965). Sodium azide was added to a final concentration of 0.02%. Final haemoglobin concentrations were usually 14 mg. protein/ml. in 0.01 M TRIS-HCl pH 7.5.

Modifications to the assay were that 0.3 ml. of substrate and

1.2 ml. of 0.01 M TRIS-HCl buffer were mixed, preincubated at 37°C for 15 min., and then 0.01-0.05 ml. samples to be tested for proteolytic activity were added at 15 second intervals. After 15 min. at 37°C, 1 ml. volumes of cold 5% TCA were added to each tube also at 15 second intervals. These were kept at 4°C for 10 min., centrifuged for 15 min. at 27,000 X g and then the A 280 nm. of the supernatants were read.

One unit of enzyme is defined as that amount which causes an increase in A 280 nm. of 1.0 in the acid-soluble fraction.

3) Casein Assay: i) Preparation of Reagent: For this assay, the method of Kunitz (1946) was used with the following modifications: Five gm. of casein were added to 400 ml. of 0.01 M TRIS-HCl buffer pH 7.5 with 0.05% sodium azide. This was agitated at 300 rpm. for 4 hours at 4°C. The mixture was then centrifuged at 27,000 X g for 30 min., and the supernatant kept. The latter had its pH adjusted to 7.5 with 1 M NaOH. Final casein concentrations were routinely 3 mg. protein/ml. The only exception to the above procedure was for the determination of pH optima in which 0.02 M TRIS (no HCl) was used. The final concentration for the latter was 9 mg. protein/ml.

ii) Protease Assay: The procedure to determine proteolytic activity is similar to the haemoglobin assay with the exception that 1.5 ml. of the casein reagent, rather than 0.3 ml. haemoglobin and 1.2 ml. buffer, was used. One unit of enzyme is defined as that amount which caused an increase in A 280 nm. of 1.0 for the acid-soluble fraction.

4) Collagen Assay: The preparation, sterilization, and test procedure with steerhide collagen followed the method by Giffee *et al.* (1965). Collagen of 4-5 mg. was preincubated for 15 min. at 37°C

in 0.9 ml. of 0.01 M TRIS-HCl pH 7.5. 0.10 ml. of either culture supernatant or purified enzyme was added, and then the samples were incubated for an additional 3 hrs. at 37°C with occasional mixing. The buffer was then removed, 1 ml. of 0.3 M $CaCl_2$ added, and the tube then incubated at 55°C for 15 min.

Collagenase activity was estimated visually by estimating the solublization of the collagen block.

VIII Determination of Protein Concentrations:

To determine protein concentration, the technique of Lowry *et al.* (1951) was used. Crystallized and lyophilized bovine serum albumin (Sigma Chemical Company) was used for the standard. Final mixtures were read for absorbance at 660 nm.

IX Discontinuous Gel Electrophoresis:

Polyacrylamide gels of pH 8.6 and 4.3 were prepared and used according to the methods of Davis (1964) and Reisfield *et al.* (1962) respectively, as modified by Shuster (1971). The reagents are listed on the following pages.

0.5 cm. (inner diameter) by 7 cm. glass tubes had 1.0 ml. small pore and 0.2 ml. large pore gel dispensed into each. These were prerun for 2 hrs. at 2 mA/gel tube, the chamber buffers were replaced and the gels were then left to cool for 30-60 min.

Samples were prepared by the addition of crystalline sucrose to a final concentration of 10%. To gels of the system pH 8.6, 5 μl of 0.05% bromophenol blue were added to each sample; to those of pH 4.3, 0.05 ml. of methyl green was added to a separate gel. As before,

REAGENTS FOR DISCONTINUOUS GEL ELECTROPHORESIS AT pH 8.6

Buffer for Separating Gels (pH 8.6)

1 N HCl	48 ml.
TRIS	36.6 gm.
TEMED	0.23 ml.

water to 100 ml.

Buffer for Spacer Gels (pH 6.7)

1 N HCl	48 ml.
TRIS	5.98 gm.
TEMED	0.46 ml.

water to 100 ml.

Small Pore Reagent

Acrylamide	28.0 gm.
BIS	0.74 gm.

water to 100 ml.

Large Pore Reagent

Acrylamide	10.0 gm.
BIS	2.5 gm.

water to 100 ml.

Riboflavin: 4.0 mg./100 ml. water

Sucrose: 40.0 gm./100 ml. water

Ammonium persulphate (fresh): 0.14 gm./100 ml. water

Chamber Buffer (pH 8.3)

TRIS	6.0 gm.
Glycine	28.8 gm.

water to 1 litre (dilute above 1:10 with water before use)

Tracking Dye

0.05% Bromophenol Blue run on same gels as samples

Stock solutions were prepared with double-distilled water, filtered, then stored in brown glass bottles at 4°C.

Terminals: Cathode (-) upper electrode
Anode (+) lower electrode

REAGENTS FOR DISCONTINUOUS GEL ELECTROPHORESIS AT pH 4.3

Buffer for Separating Gels (pH 4.3)

1 N KOH	48.0 ml.
Glacial acetic acid	22.4 ml.
TEMED	4.6 ml.

water to 100 ml.

Buffer for Spacer Gels (pH 5.8)

1 N KOH	48.0 ml.
Glacial acetic acid	2.88 ml.
TEMED	0.46 ml.

water to 100 ml.

Small Pore Reagent

Acrylamide	28.0 gm.
BIS	0.74 gm.

water to 100 ml.

Large Pore Reagent

Acrylamide	10.0 gm.
BIS	2.5 gm.

water to 100 ml.

Riboflavin: 4.0 mg./100 ml. water

Sucrose: 40.0 gm./100 ml. water

Ammonium persulphate (fresh):
0.4 gm./100 ml. water

Chamber Buffer (pH 4.5)

β alanine	62.4 gm.
Glacial acetic acid	16.0 ml.

water to 1 litre
(dilute above 1:10 with water before use)

Tracking Dye

0.05% Methyl Green run on gels other than those used for samples

Stock solutions were prepared with double-distilled water, filtered, then stored in brown glass bottles at 4°C.

Terminals: Anode (+) upper electrode
Cathode (-) lower electrode

electrophoresis was at 2 mA/tube and until the tracking dye was 2-5 mm. from the end of its tube. Gels were stained 1 hour in 1% Buffalo Black in 7% acetic acid (Racusen, 1973; Wilson, 1973). Gels were destained electrophoretically for 20 min. in 7% acetic acid.

For the location of proteolytic activity, duplicate gels were run concurrently. One was stained as described above, the other was cut into 2 mm. transverse slices, each slice placed into a 125 ml. Erlenmeyer flask with 10 ml. of 0.01 M TRIS-HCl buffer pH 7.5 and 0.05% sodium azide. The samples were dispersed with a glass rod, then 25 mg. of azocoll were added and the flasks incubated at 30°c for 12 hours with shaking. The contents of the flask were then filtered and the A 580 nm. of the filtrate measured.

X SDS-Slab-Gel Electrophoresis:

The procedure of Ames (1974) was followed, using the slab-gel electrophoresis apparatus, Model SE 520 (Hoefer Scientific Instruments) with gel dimensions of 28 cm. X 14 cm. X 1.5 mm.. The reagents used were as listed on the following page. The determination of molecular weights of SDS-protein complexes was as described by Neville (1971).

The separating gel was prepared first, dispensed into the slab gel apparatus, and then overlayed with a thin film of 1-butanol. After polymerization, the surface of the gel was rinsed with distilled water containing 0.1% SDS, and then the stacking gel was added.

Protein samples were dialyzed against distilled water, lyophilized, and finally suspended in sample buffer. Electrophoresis was carried out with an initial current of 5 mA until the bromophenol blue was concentrated at the interphase of the stacking and separating gels.

REAGENTS FOR SDS-SLAB-GEL ELECTROPHORESIS

Reagents	Volume (ml.) Separating Gel (pH 8.8) (12.5% acrylamide)	Stacking Gel (pH 6.8) (5% acrylamide)
M TRIS-HCl pH 8.8	30.00	-
M TRIS-HCl pH 6.8	-	2.50
10% SDS	0.80	0.20
30% acrylamide, 0.85% BIS	32.41	3.24
TEMED	0.06	0.01
Distilled water	16.13	13.95
10% ammonium persulphate (fresh)	0.60	0.10
TOTAL	80.00	20.00

Electrode Buffer	Volume (ml.)
10 fold strength TRIS-glycine (30.3 gm. TRIS, 144 gm. glycine in one litre water)	200
10% SDS	20
Distilled water	1780
TOTAL	

Sample Buffer	Volume (ml.)
M TRIS-HCl pH 6.8	0.6
10% SDS	2.0
5% 2-mercaptoethanol	0.5
20% Glycerol	2.0
0.01% Bromophenol Blue	0.1
Distilled water	4.8
TOTAL	10.0

Stock solutions were prepared with double-distilled water, filtered, then stored in brown glass bottles at 4°C.

The current was then increased to 12.5 mA and left until the tracking dye reached the bottom of the gel.

The proteins were fixed and stained by flooding the removed gel with a solution of 25% 2-propanol, 10% acetic acid, and 0.05% Coomassie Blue. Destaining was by several changes of 25% 2-propanol in 10% acetic acid.

RESULTS AND DISCUSSION

I) PRODUCTION OF PROTEASE ACTIVITY BY PSEUDOMONAS 461-3-11

1) Evaluation of Protease Assays:

During the course of the investigation on Pseudomonas 461-3-11, three assays were used to determine proteolytic activity in cell cultures, crude supernatants, or purified enzyme preparations. Generally, the haemoglobin or casein substrates were used if several samples had to be analyzed, while azocoll was used if higher levels of sensitivity were required. Figure 1 is a comparison of the hydrolysis rates of the crude protease on the three substrates.

i) The Azocoll Assay: This procedure had the advantage of maximum sensitivity. The method of Moore (1969) was followed despite several authors reporting maximum absorbance at 520 nm. (Oakley et al., 1946; Todd et al., 1948; Boethling, 1975) rather than 580 nm. (Jackson and Matsueda, 1970). Figure 2 is an absorption spectrum of the product released from azocoll by crude Pseudomonas 461-3-11 protease. Although the maximum absorbance was found to be at 520 nm., to be consistent with initial experiments, the A 580 nm. was used throughout.

ii) The Haemoglobin Assay: Figure 1 reveals that haemoglobin is less readily degraded than either casein or azocoll. It should be noted that, at the end of 2 hours, although casein and haemoglobin were present in an initial concentration of 3 mg./ml., the latter was less readily hydrolyzed, as evidenced by the smaller amount of TCA soluble material released by digestion with crude protease. This difference in rates of hydrolysis on various substrates has been

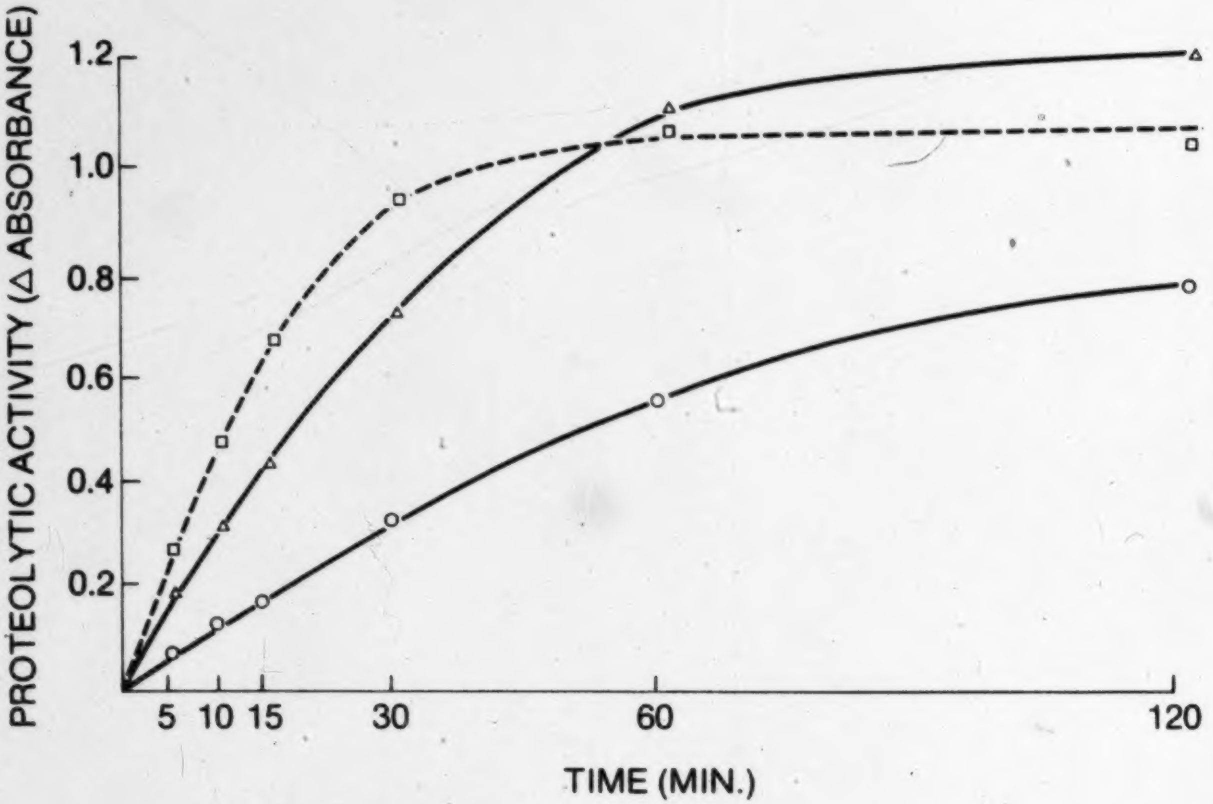

PROTEOLYTIC ACTIVITY (△ ABSORBANCE)
1.2
1.0
0.8
0.6
0.4
0.2
5
10
15
30
60
120
TIME (MIN.)

FIGURE 1

PROTEOLYTIC ACTIVITY OF CRUDE PSEUDOMONAS 461-3-11 PROTEASE ON THREE SUBSTRATES

Three substrates were preincubated at 37°C for 15 minutes (see Materials and Methods). 0.01 ml. of a 24 hour *Pseudomonas* 461-3-11 culture supernatant was then added. The concentration of casein and hemoglobin were both 3 mg./ml., that of azocoll 5 mg./ml.. All were in 0.01 M TRIS-HCl buffer pH 7.5.

Activity on Haemoglobin, Δ A 280 nm. ○———○
Activity on Casein, Δ A 280 nm. △———△
Activity on Azocoll, Δ A 580 nm. □-------□

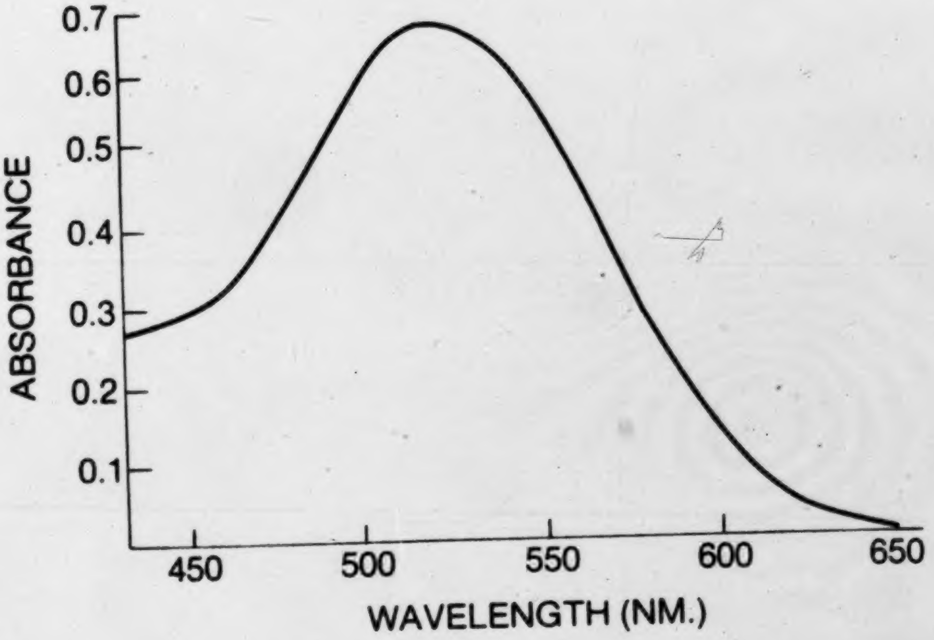

0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE
450
500
550
600
650
WAVELENGTH (NM.)

FIGURE 2

THE ABSORBANCE SPECTRUM OF THE HYDROLYSIS PRODUCTS OF AZOCOLL RELEASED BY CRUDE PSEUDOMONAS 461-3-11 PROTEASE

0.28 units of crude protease were added to 25 mg. of azocoll in 5 ml. of 0.01 M TRIS-HCl buffer pH 7.5 (see Materials and Methods). After 15 minutes at 37°C, the mixture was filtered and the filtrate then scanned on a Unicam SP 8000 Spectrophotometer.

observed with other proteases. *Pseudomonas lachrymans* can degrade haemoglobin but not gelatin (Keen *et al.*, 1967), an arctic isolate of *Pseudomonas* can hydrolyze gelatin but not bovine serum albumin (McDonald *et al.*, 1963), while a *Serratia sp.* can hydrolyze only denatured lysozyme, insulin or IgG (Miyata *et al.*, 1970b).

iii) The Casein Assay: This substrate, although initially clear (see Materials and Methods for its preparation), becomes opalescent upon hydrolysis (Skelton *et al.*, 1977). Casein normally exists in milk as micelles formed by the interaction of αS1, β, and κ casein (Pepper *et al.*, 1970) and stabilized by calcium ions (Chiba *et al.*, 1970). Cleavage of the κ-casein, which is also essential for stability, releases a fragment that is soluble in 12% TCA, leaving the rest of the protein which is largely insoluble in water (Armstrong *et al.*, 1966; Sternberg, 1972). Whether the proteases of *Pseudomonas* 461-3-11 followed the same mechanism was not investigated. Clearing of the opalescent solution under continued incubations can be accounted for by the further hydrolysis of all casein peptides.

The addition of 0.02% sodium azide to all preparations to prevent microbial growth was done on the assumption that this reagent did not affect protease activity. The mechanism of microbial inhibition by sodium azide was originally believed to inhibit oxidative phosphorylation (Roisin and Kepes, 1973) by binding to cytochromes (Wever *et al.*, 1973a; Wever *et al.*, 1973b). It has been shown, however, to interact with enzyme systems unrelated to respiration [e.g. guanulate cyclase (Kimura *et al.*, 1976), threonine synthetase (Daniel, 1976), or protein synthesis through methionine starvation (Nazar and Wong, 1972; Nazar and Wong, 1973)]. Whether this would play a role in this study is unclear.

2) Factors Affecting Growth and Enzyme Production:

i) Phase of Growth: To determine when the protease appeared in the growth medium, 300 ml. of 3% TCS in a 2 litre flask was inoculated, incubated at 23°C with an agitation of 300 rpm., and at the noted times 2 ml. aliquots were removed for various assays. Proteolytic activity (azocoll assay), total and viable cell number (Materials and Methods) were obtained for each sample.

Despite difficulties in determining total cell number, this count does approximately correspond to viable number. As is evident from Table 1, proteolytic activity was not a constant function of cell number. During the initial 9 hours, none of the former was detected while from 15 hours to 20 hours after inoculation, proteolytic activity increased 250% to an increase in cell number of 25%. Enzyme production, therefore, was found to occur near the end of the culture's growing phase.

As previously mentioned within the introduction of this text, extracellular enzymes usually appear in the media during the exponential phase of growth. Release of these enzymes is by a mechanism other than cell autolysis (Winters and Corpe, 1971). Within the genus Pseudomonas, cell autolysis has been observed, but only after the cells have entered stationary phase (Levin and Sickle, 1976; Rogul and Carr, 1972).

ii) Temperature: Table 2 shows the effect of various incubation temperatures on Pseudomonas 461-3-11 (see footnote of Figure 3). The exponential growth-rate constants or generations per hour was determined by using the equation:

TABLE 1

GROWTH OF PSEUDOMONAS 461-3-11 AND PROTEOLYTIC ACTIVITY

Time (hrs.)	OD 600 nm.	[1]Total Cell Number/ml.	[2]Viable Cell Number/ml.	[3]eu./ml.
0	0.08	3.4×10^{8}	3.3×10^{8}	0
3	0.34	1.1×10^{9}	5.2×10^{8}	0
6	2.03	4.0×10^{9}	3.1×10^{9}	0
9	4.65	9.5×10^{9}	7.0×10^{9}	0
12	7.30	3.0×10^{10}	1.9×10^{10}	0.9
15	8.64	3.8×10^{10}	3.6×10^{10}	6.2
20	10.70	4.8×10^{10}	4.5×10^{10}	22.0
25	12.85	5.6×10^{10}	6.2×10^{10}	31.4
41	15.10	6.8×10^{10}	9.6×10^{10}	41.7

1. Plate Count Assay
2. Petroff-Hauser Assay
3. Azocoll Assay

TABLE 2

GROWTH OF PSEUDOMONAS 461-3-11 AT VARIOUS TEMPERATURES

Incubation Temperature (°C)	Time (hrs.)				
	0	1.5	3.0	4.5	6.0
22.5	.033	.060	.143	.338	.746
25.0	.033	.067	.201	.405	1.29
27.5	.033	.119	.295	1.29	2.72
30.0	.033	.119	.423	1.66	3.81
32.5	.033	.147	.546	1.96	4.32
35.0	.033	.128	.531	2.08	4.10
37.5	.033	.083	.336	.866	1.84
40.0	.033	.052	.189	.259	.472
42.5	.033	.044	.075	.127	.150

Growth is expressed as OD 600 nm.

$$k = \frac{\log_{10}N_t - \log_{10}N_o}{0.301\ t}$$ (Stanier *et al.*, 1970)

where k = the number of generations per hour

N_o = population size at a certain time

N_t = population size after a subsequent time

t = duration of time between the above two populations

For these calculations, it was assumed that an OD 600 nm. of 1.0 represented 3.7 X 10^9 cells/ml. (see Table 1). Aside from temperature, growth can be restricted by other parameters such as pH, aeration, depletion of nutrients, or the accumulation of toxic end products. However, as all calculations were based on samples taken within the early phase of growth, these latter factors were judged to be roughly equivalent between the samples.

From Figure 3, it was determined that the optimum temperature for growth with 3% TCS was between 33 and 34°C.

Figure 4 indicates the kinetics of growth (OD 600 nm.) for *Pseudomonas* 461-3-11 at different temperatures. Although cultures incubated at 26, 28, and 30.5°C were also monitored, these are not presented as they were similar to 33.5°C except for a slower growth rate. Above the optimum of 33.5°C, the growth curves are unusual in that they appear synchronous, entering stationary phase at the same time. Even the pH changes in the various cultures were comparable (e.g. the initial pH was 7.21 for all, at 12 hours it was 8.0-8.1). The reasons for these similarities was not determined.

Of particular interest is the lag towards the end of the growing

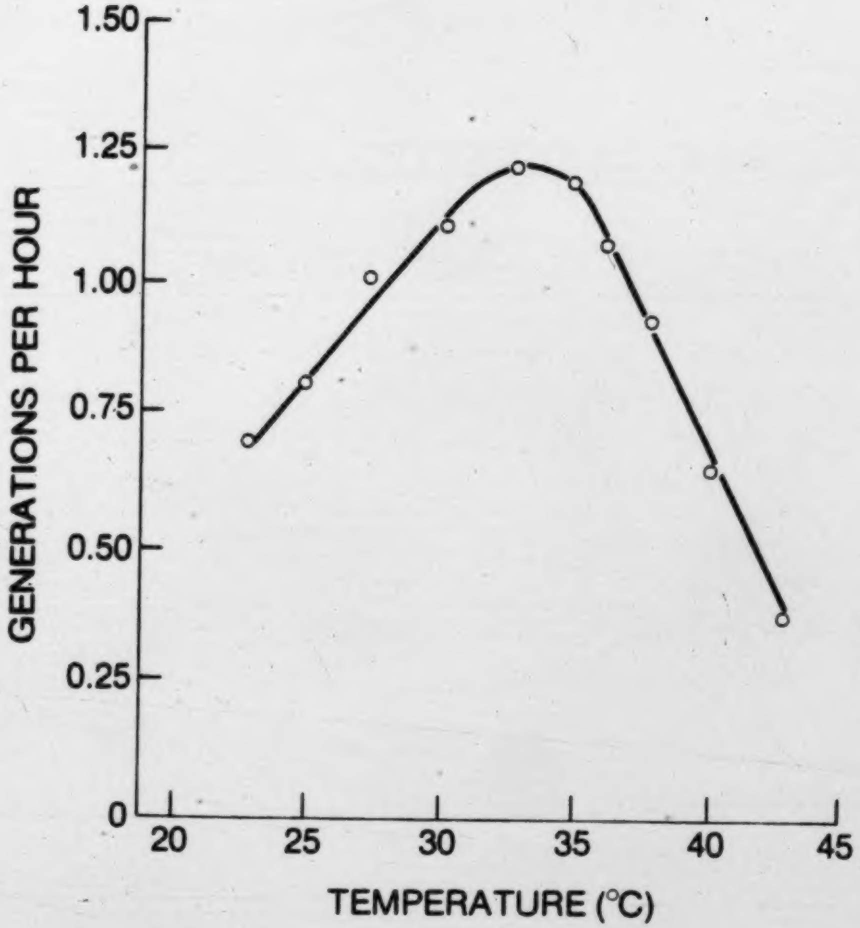

GENERATIONS PER HOUR
1.50
1.25
1.00
0.75
0.50
0.25
0
20
25
30
35
40
45
TEMPERATURE (°C)

FIGURE 3

RELATIVE GROWTH RATES OF PSEUDOMONAS 461-3-11 AT DIFFERENT TEMPERATURES

TCS was inoculated then aseptically dispensed (10 ml. per 50 ml. Erlenmeyer flask). These were then incubated in water-bath shakers at the indicated temperatures. At given times a flask from each series was removed, and growth was estimated by measuring the OD 600 nm.

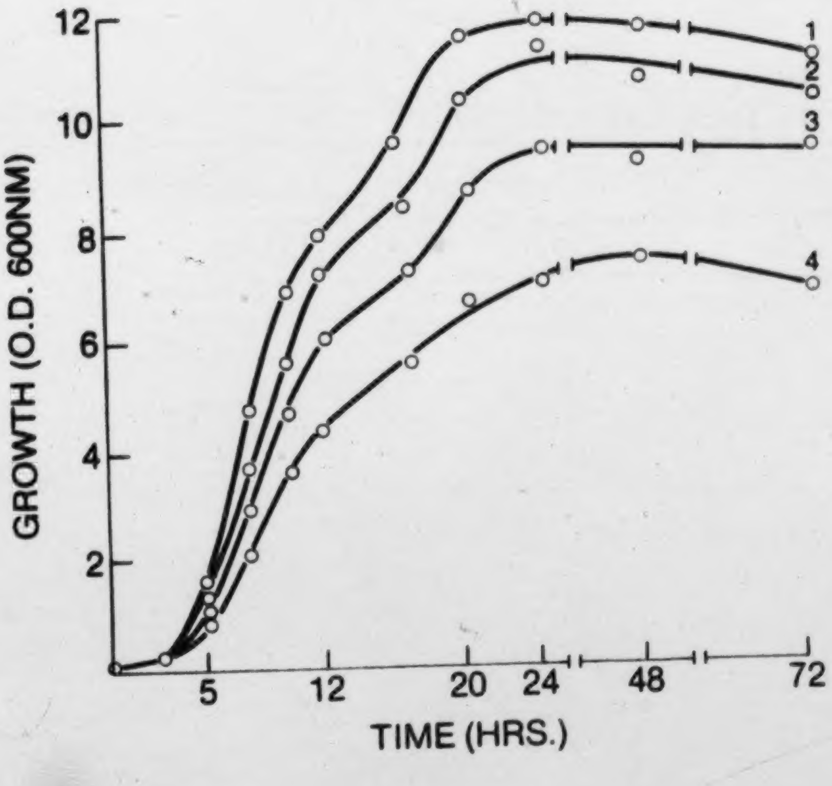

GROWTH (O.D. 600NM)
12
10
8
6
4
2
5
12
20
24
48
72
TIME (HRS.)
1
2
3
4

FIGURE 4

GROWTH OF PSEUDOMONAS 461-3-11 AT DIFFERENT TEMPERATURES

1.0 ml. of a 24 hour culture was used to inoculate 500 ml. of 3% TCS which was incubated at 26°C with shaking for one hour. 10 ml. were then dispensed into each sterilized 50 ml. Erlenmeyer flask. These were incubated at the indicated temperatures. At noted times, a flask was removed and its contents assayed for growth (OD 600 nm.) and proteolytic activity (casein assay).

OD 600 nm. Curve	Incubation Temperature (°C)
1	33.5
2	35.0
3	36.5
4	38.0

phase that is evident at 20 hours in Figure 4 (also at the 21 hour readings for growth curves 2 and 3 in Figure 6). As reviewed by Stanier *et al*. (1970), this change in the rate of growth may be due to the exhaustion of one metabolite and the subsequent utilization of another after a delay due to the synthesis of inducible catabolic enzymes. As observed by Stinson and Merrick (1974), a metabolite may be present but gradually be made unavailable to the cell. In their example of *Pseudomonas lemoignei*, an increase in the culture's pH blocked the transport mechanism for succinate which then led to a derepression of protease production of the cell. Whether a similar mechanism also occurred for *Pseudomonas* 461-3-11 was not researched.

Figure 5 presents the proteolytic activity of the cultures of *Pseudomonas* 461-3-11 at different temperatures. Although at the peak of enzyme production proteolytic activity at 26°C was approximately 50 fold greater than at 38°C, the OD 600 nm. of the former culture was only about twice that of the latter (see Figure 4).

Juffs *et al.* (1968) noted that for several *Pseudomonas spp.*, proteolytic activity per cell was far greater at 3°C than at 28°C, while Kato *et al.* (1972) found that protease production of a marine psychrophile decreased greatly as the temperature increased. *Pseudomonas* 461-3-11, therefore, resembles several others in being more efficient in the production of its protease at suboptimal temperatures.

Twenty-eight degrees gave the highest yields but due to the loss of activity of the enzyme (see Figure 5) and for convenience, incubation for the production of the protease in subsequent experiments was undertaken at room temperature (22-26°C).

Upon reviewing Figure 5, the most apparent characteristic is the

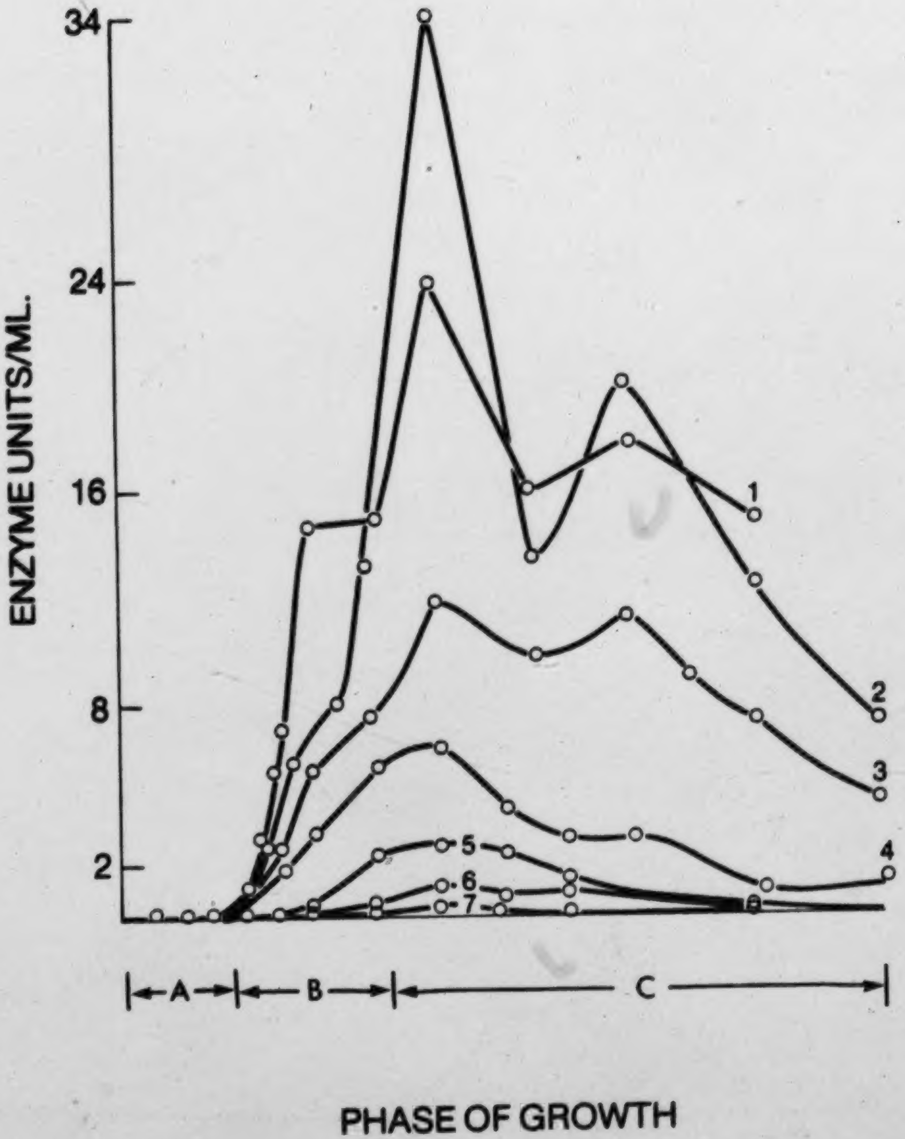

34
24
16
8
2
ENZYME UNITS/ML.
1
2
3
4
5
6
7
A
B
C
PHASE OF GROWTH

FIGURE 5

KINETICS OF PROTEOLYTIC ACTIVITY OF PSEUDOMONAS 461-3-11 AT DIFFERENT TEMPERATURES

For clarity, proteolytic activities have been so arranged as to correspond with stages of growth. Enzyme activity was determined by the casein assay.

Proteolytic Activity Curve	Incubation Temperature of Culture (°C)
1	26.0
2	28.0
3	30.5
4	33.5
5	35.0
6	36.5
7	38.0

Phase of Growth: A = Increasing Growth Rate
B = Decreasing Growth Rate
C = Stationary-Decline

undulate nature of enzyme production. Friedman et al. (1953) reported similar findings for Bacterium linens proteolytic activity, while aside from different temperature ranges, almost identical results were reported by Peterson and Gunderson (1960) in their investigations with Pseudomonas fluorescens. Only the former authors ventured the speculation that the undulate nature was due to the production of two different enzymes. As noted in the introduction of this thesis, Aeromonas proteolytica produces an aminopeptidase peak first and then only after several hours does the endopeptidase peak appear (Prescott and Wilkes, 1966). The sections dealing with purification and characterization of Pseudomonas 461-3-11 will lend support to the hypothesis that the undulate nature of proteolytic activity is due to the production of different enzymes.

iii) Degree of Aeration: When research was first undertaken, attempts to increase the culture volume and hence yield of protease appeared counterproductive. To determine if aeration was the contributing factor, 700 ml. of 3% TCS was inoculated, shaken thoroughly and then dispensed as six 10 ml., two 50 ml., and one 400 ml. volumes in the appropriate flasks. All were incubated at 30°C with agitation at 300 rpm.. Growth response (OD 600 nm.) and enzyme production (haemoglobin assay) were monitored at given intervals using 1 ml. samples. One 10 ml. culture was used per sampling time, one 50 ml. culture was used three times before proceeding to the next, and the one 400 ml. culture was used throughout the experiment.

Table 3 shows the results of different surface to volume ratios (degree of aeration) on growth and enzyme production of Pseudomonas

461-3-11. For the sake of being concise, only the data for 16 hours has been presented.

TABLE 3

THE EFFECT OF DIFFERENT CULTURE VOLUMES ON GROWTH AND ENZYME PRODUCTION BY PSEUDOMONAS 461-3-11

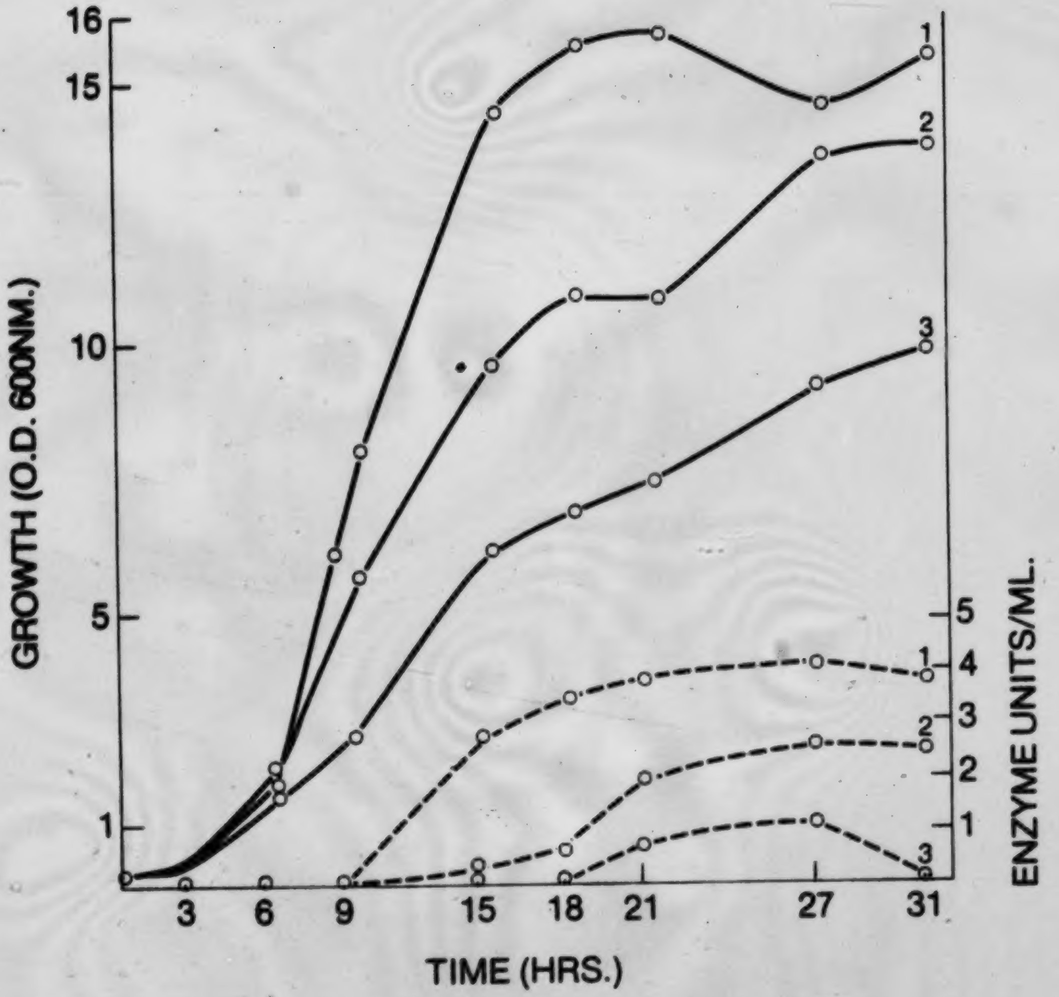
Volume of Culture Dispensed	Final pH	OD 600 nm.	eu./ml.[1]
10 ml./50 ml. flask	8.21	11.17	4.6
50 ml./250 ml. flask	8.00	8.24	2.6
400 ml./2 litre flask	7.62	9.17	2.0

1. Haemoglobin assay

Attempts to scale up to 5 litres of culture using the Microfermentor (see Materials and Methods) were even less productive, yielding a peak of 1.4 eu./ml. at the end of 17 hours.

Although the ratios of culture volumes to flask sizes were constant in Table 2, the lower surface per volume of larger cultures appears to have impeded aeration. As for the Microfermentor, the air flow of 5 litres/min. with an agitation of 300 rpm. was also clearly suboptimal. Following the procedure of Kato *et al.* (1972), Figure 6 confirms that culture size restricts aeration and hence growth or enzyme production. A comparison of curves 1 and 3 for proteolytic activity reveals that the effect of poor aeration is not to delay but rather to decrease enzyme production.

Several authors have observed a similar correlation between extracellular enzyme production and aeration. Coleman and Elliott (1965)



GROWTH (O.D. 600NM.)
16
15
10
5
1
ENZYME UNITS/ML.
5
4
3
2
1
1
2
3
1
2
3
3
6
9
15
18
21
27
31
TIME (HRS.)

FIGURE 6

THE EFFECTS OF THE DEGREE OF AERATION ON CULTURES OF PSEUDOMONAS 461-3-11

One litre of 3% TCS within a 2 litre flask was inoculated with 2 ml. of a 24 hour culture of Pseudomonas 461-3-11. This was shaken at 300 rpm. at room temperature (26°C) for one hour, then the volumes noted below were dispensed into 1 litre Erlenmeyer flasks. Incubation was as described in the text. At noted times, 1 ml. aliquots were removed and analyze for growth (OD 600 nm.) and proteolytic activity (haemoglobin assay).

	Growth (OD 600 nm.)	Proteolytic Activity
50 ml. culture	o———o 1	o- - - - - - - -o 1
250 ml. culture	o———o 2	o- - - - - - - -o 2
500 ml. culture	o———o 3	o- - - - - - -o 3

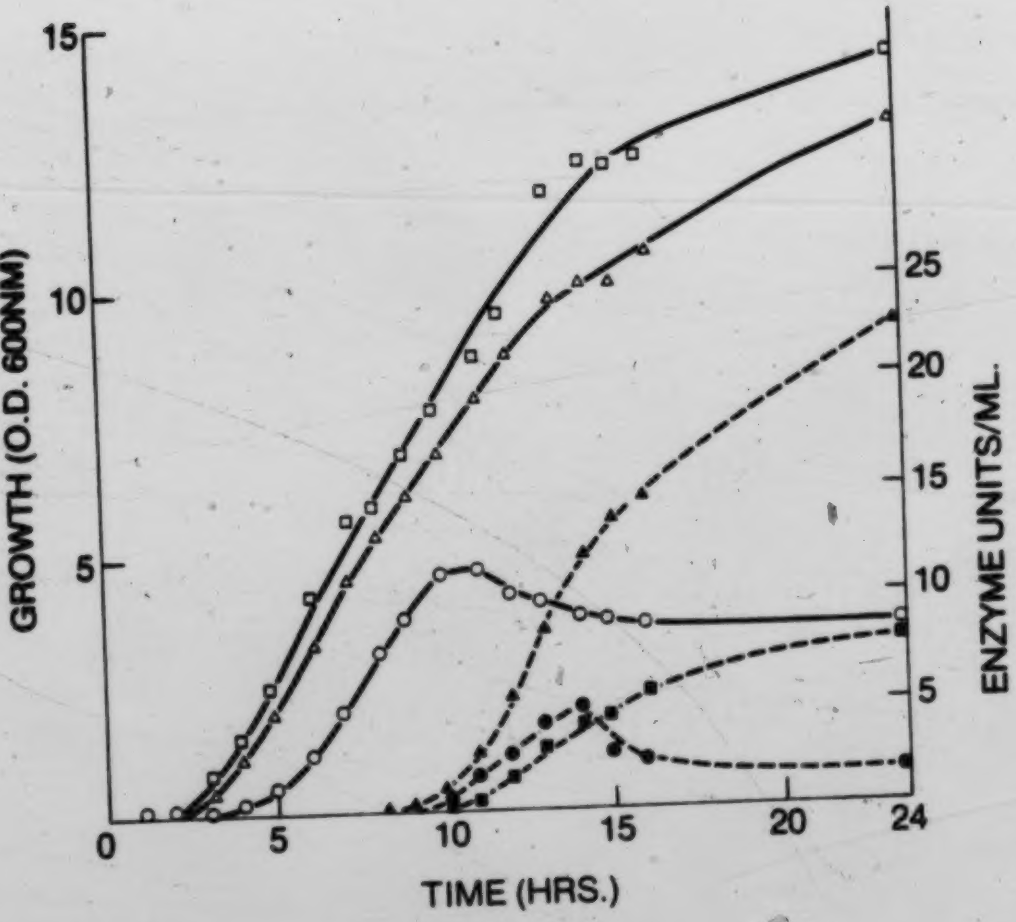
noted that the production of ribonuclease by Bacillus subtilis was inhibited by anaerobiosis, Keen and Williams (1967) reported the influence of aeration on both growth and the production of a proteinase by Pseudomonas lachrymans, while Kato et al. (1972) found that as the culture volume increased within a 500 ml. flask, proteolytic activity decreased for a psychrophilic Pseudomonas sp.

Although aeration plays a significant role in enzyme production as noted in Figure 6, the actual mechanism as yet is unclear. For the growth of some Pseudomonas spp., a decrease of oxygen to 0.005 atmosphere has not been found significant (Pierson et al., 1970; Clark and Burki, 1971) while the increase of carbon dioxide to 0.3 atmosphere is of little consequence to growth when temperatures are above 20°C (Ingram, 1962; Clark and Lentz, 1969).

iv) Growth Media: Growth response and protease production of Pseudomonas 461-3-11 were tested in nutrient broth, brain-heart infusion, and trypticase soy broth. The results are summarized in Figure 7 and details are given in the accompanying legend.

By far TCS gave the best results for proteolytic activity while brain-heart infusion gave the highest growth response. Nutrient broth supported the least amount of growth or proteolytic activity. The media constituents responsible for these differences were not determined.

Several attempts to grow Pseudomonas 461-3-11 in various synthetic media proved unsuccessful. The medium by Eyzaguirre et al. (1973), although suitable for Pseudomonas aeruginosa, proved inadequate for the former bacterial species despite supplements of minerals, amino acids, or carbohydrates (see Appendix). As yeast extract and vitamin-free casamino acids did stimulate growth, an organic nitrogen source was



GROWTH (O.D. 600NM)
15
10
5
0
5
10
15
20
24
TIME (HRS.)
ENZYME UNITS/ML.
25
20
15
10
5

FIGURE 7

GROWTH AND PROTEOLYTIC ACTIVITY OF PSEUDOMONAS 461-3-11 IN DIFFERENT MEDIA

The microbe was transferred three times in the noted broths, then 0.1 ml. of a 24 hour culture was used to inoculate 200 ml. of the respective media within a 1 litre flask. Incubation was at 30°C with shaking. Proteolytic activity was determined by the azocoll assay.

	Growth (OD 600 nm.)	Proteolytic Activity
Nutrient Broth	○———○	●- - - - -●
Brain-Heart Infusion	□———□	■- - - - -■
Trypticase Soy Broth	△———△	▲- - - - -▲

the probable requirement. The specific nature of this requirement was not investigated.

In 1964, Merkel et al. reported different proteolytic activities and growth responses of Aeromonas proteolytica on simply two different lot numbers of Difco peptone. In the introduction of this thesis, also, it was noted that several researchers reported that either proteins or their partial hydrolysates stimulate the production of proteases by Pseudomonas spp.. The partial hydrolysates of proteins within TCS, therefore, may also be stimulatory for the enzyme production by Pseudomonas 461-3-11. Other contributing factors within TCS aside from those listed on the label are the denatured trypsin of 24,000 molecular weight, carmalized components, and Maillard products (personal communications, B. M. Smith, Technical Services of Baltimore Biological Laboratories, 1975).

From the results of Figure 7, TCS was chosen as the medium for the production of protease. On a number of occasions, however, this proved a great source of anxiety as cultures would tend to lose their proteolytic activity after prolonged subculturing in this medium. This then necessitated starting anew with a lyophilized active stock culture. This spontaneous loss on TCS or gelatin-agar that had been supporting growth for several weeks appears similar to the observations of McKay and Baldwin (1975) or Efstathiou and McKay (1976) for extracellular proteinase activity in Streptococcus lactis. In these studies, the spontaneous loss of enzyme activity led to the eventual correlation between the production of protease and a 10-million-dalton plasmid. Possibly similar lines of research may prove a rewarding avenue of research for the extracellular proteases of Pseudomonas 461-3-11.

II) PURIFICATION OF PSEUDOMONAS 461-3-11 EXTRACELLULAR PROTEASES

The purification sequence which proved the most satisfactory is summarized in the following flow sheet:

PURIFICATION SEQUENCE FOR PSEUDOMONAS 461-3-11 PROTEASES

Grow cells in TCS for 36 hours at 22°C

↓

Centrifuge culture at 27,000 X g for 30 minutes. Discard cells. The supernatant is "crude enzyme".

↓

Add ammonium sulphate to 36% saturation at 4°C.

↓

Keep at 4°C for 8 hours. Centrifuge at 27,000 X g for 30 minutes. Discard pellet.

↓

Add ammonium sulphate to the supernatant to 65% saturation at 4°C.

↓

Keep at 4°C for 8 hours. Centrifuge at 27,000 X g for 30 minutes. Discard supernatant.

↓

Redissolve pellet in distilled water. Dialyze against 0.01 M TRIS-HCl buffer pH 8.0 at 4°C for 8 hours. Centrifuge at 27,000 X g for 15 minutes. Discard pellet.

Apply supernatant to a 2.5 cm. X 35 cm. column of DEAE-cellulose, 4°C. Elute with a 0-1.0 M NaCl gradient in 0.01 M TRIS-HCl buffer pH 8.0.

↓

Concentrate the active peak of proteolytic activity by PEG at 4°C.

↓

Dialyze against 0.01 M NaH_2PO_4-Na_2HPO_4 buffer pH 6.0 for 8 hours at 4°C.

↓

Apply to a 2.5 cm. X 35 cm. column of CM-cellulose, 4°C. Elute with a 0-0.3 M NaCl gradient in 0.01 M NaH_2PO_4-Na_2HPO_4 buffer.

Protease 1 does not adsorb to the column.
Protease 2 elutes with 0.03 M NaCl.
Protease 3 elutes with 0.07 M NaCl.

↓

Test purity by disc gel electrophoresis.

The individual steps in this sequence will be discussed separately in more detail.

1) Crude Supernatant:

Maximum enzyme production in 200 ml. of TCS in 2 litre flasks, occurred between 24-36 hours incubation at 23°C, 300 rpm.. These conditions were routinely used for the production of Pseudomonas 461-3-11 proteases for purification. Cells were removed by centrifugation at

27,000 X g for 30 minutes.

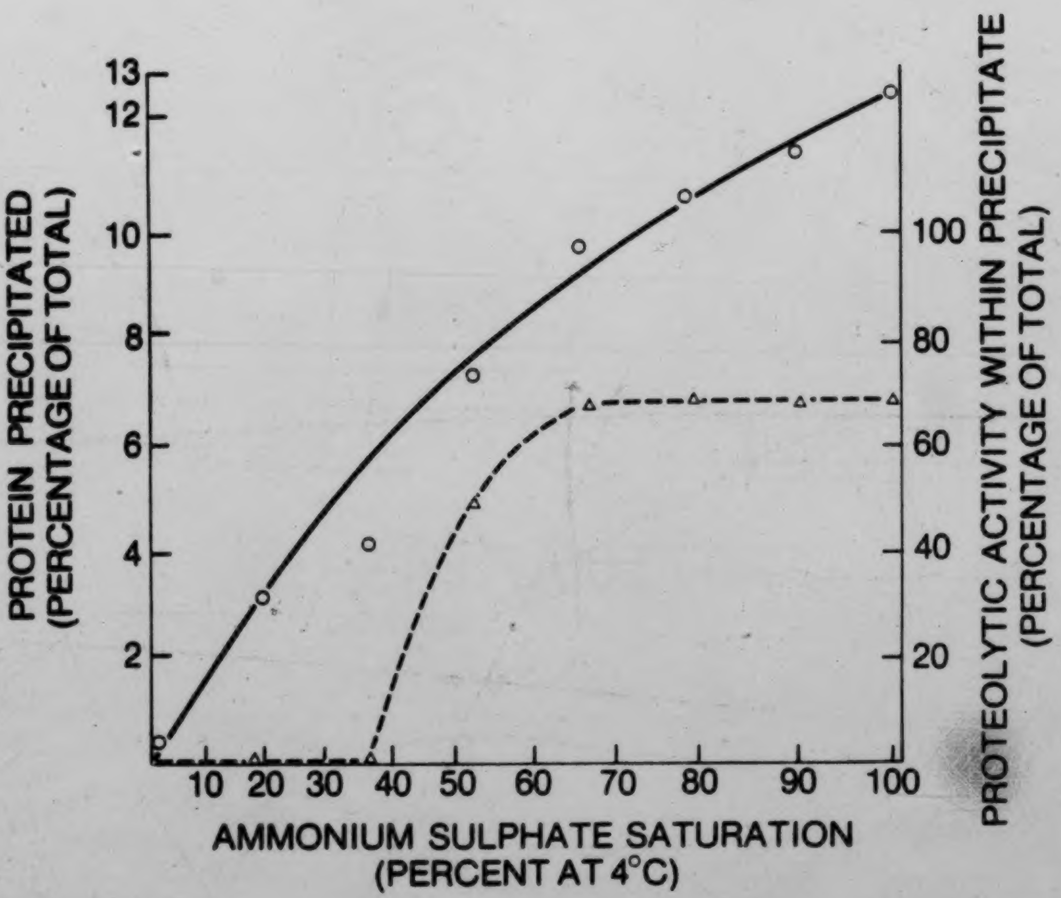
2) Ammonium Sulphate Precipitation:

The crude supernatant was fractionated by precipitation with ammonium sulphate as described in the legend of Figure 8. The results, as presented in Figure 8, show that approximately 70% of the activity and 5.5% of the total initial protein was precipitated at 36-65% saturation at 4°C. Below 36% saturation, 100% of the proteolytic activity was found in the soluble fraction; above 65% saturation, only 1% of the proteolytic activity remained. About 29% of the activity, therefore, was lost through inactivation by precipitation.

Precipitation by the addition of ammonium sulphate has been used to purify *Pseudomonas* extracellular proteases although with different results. Although Boethling (1975) found it suitable for the protease of *Pseudomonas maltophilia*, Carrick and Berk (1975) found the collagenase of *Pseudomonas aeruginosa* extremely sensitive to concentrations of ammonium sulphate. It was subsequently found with investigation of *Pseudomonas* 461-3-11 that the microbe produced three proteases. It was not determined, however, whether ammonium sulphate partially inactivated all or specifically acted against one enzyme.

3) Dialysis:

The precipitate from 36-65% saturation (4°C) was suspended in distilled water, 1.0 ml. was dispensed into each of several dialysis bags (1/4 inch tubing), and then dialyzed against the buffers indicated in Table 4. To compensate for a dilution of bag contents due to an uptake of water (usually an increase in volume of 40%), activity within



PROTEIN PRECIPITATED
(PERCENTAGE OF TOTAL)
13
12
10
8
6
4
2
10
20
30
40
50
60
70
80
90
100
AMMONIUM SULPHATE SATURATION
(PERCENT AT 4°C)
100
80
60
40
20
PROTEOLYTIC ACTIVITY WITHIN PRECIPITATE
(PERCENTAGE OF TOTAL)

FIGURE 8

AMMONIUM SULPHATE PRECIPITATION OF PSEUDOMONAS 461-3-11 PROTEASE FROM CRUDE CULTURE SUPERNATANT

From a 26 hour culture of *Pseudomonas* 461-3-11 grown at 26°C, the cells were removed by centrifugation at 27,000 X g for 30 min.. To 10 ml/ aliquots of the supernatant, solid ammonium sulphate was added. After being left 12 hours at 4°C, the samples were centrifuged as above, the supernatant removed, and the pellet redissolved in 10 ml. of distilled water. Protein was determined by the method of Lowry *et al.* (1951), proteolytic activity by the azocoll assay.

Protein	o———o
Proteolytic Activity	△- - - - -△

TABLE 4

THE EFFECT OF DIALYSIS ON AN AMMONIUM SULPHATE PRECIPITATED PROTEASE OF PSEUDOMONAS 461-3-11

Time of Dialysis (Hrs.)	Relative Conductivity (%)	Relative Proteolytic Activity	
		Dialyzed against 0.01 M TRIS pH 7.5	Dialyzed against 0.01 M phosphate pH 6
1	100	70	76
2	19	78	79
4	5.6	77	79
8	3.2	79	71
16	2.6	83	91
24	2.6	76	84
Buffer only	2.6	0	0

each was multiplied by:

$$\frac{\text{Initial sample protein/ml.}}{\text{Protein within dialysis bag/ml.}}$$

Proteolytic activity was determined by the azocoll assay, protein by the method of Lowry *et al.* (1951). The conductivity was measured to determine the salt content. Aside for some minor activation over several hours, neither the length of dialysis nor the ionic species of the buffer appeared to reverse the partial inactivation by ammonium sulphate precipitation. Table 4 also indicated that dialysis should be for at least 8 hours to achieve equilibrium.

Upon dialysis against 0.01 M phosphate buffer pH 6.0, a precipitate of protein was found within the dialysis bag. Since the specific activity of the soluble portion was unchanged, it was concluded that the enzyme was being precipitated. The precipitation appeared to be dependent on the concentration of protein as dilution in phosphate buffer pH 6.0 redissolved this precipitate. It should be noted that cultures of *Pseudomonas* 461-3-11 were usually at pH 8.0 or higher upon harvesting the protease.

4) DEAE-cellulose:

In order to compare the effect of pH on the binding of proteolytic activity to DEAE-cellulose, 4 samples were used as follows:

i) A 36-65% saturation ammonium sulphate precipitate was divided into 4 equal parts and each was dialyzed to equilibrium against either:

a) 0.01 M NaH_2PO_4-Na_2HPO_4 buffer pH 6.0

b) 0.01 M NaH_2PO_4-Na_2HPO_4 buffer pH 7.0

c) 0.01 M TRIS-HCl buffer pH 8.0

d) 0.01 M TRIS-HCl buffer pH 9.0

ii) Each sample was applied to a fresh 2.5 cm. X 25 cm. column of DEAE-cellulose, eluted with 120 ml. of the respective buffer, then with a 0-1.0 M NaCl gradient also in the same buffer.

iii) 6.8 ml. fractions were collected and assayed for protein (A 280 nm.), Protease activity (haemoglobin assay), and NaCl concentration (conductivity).

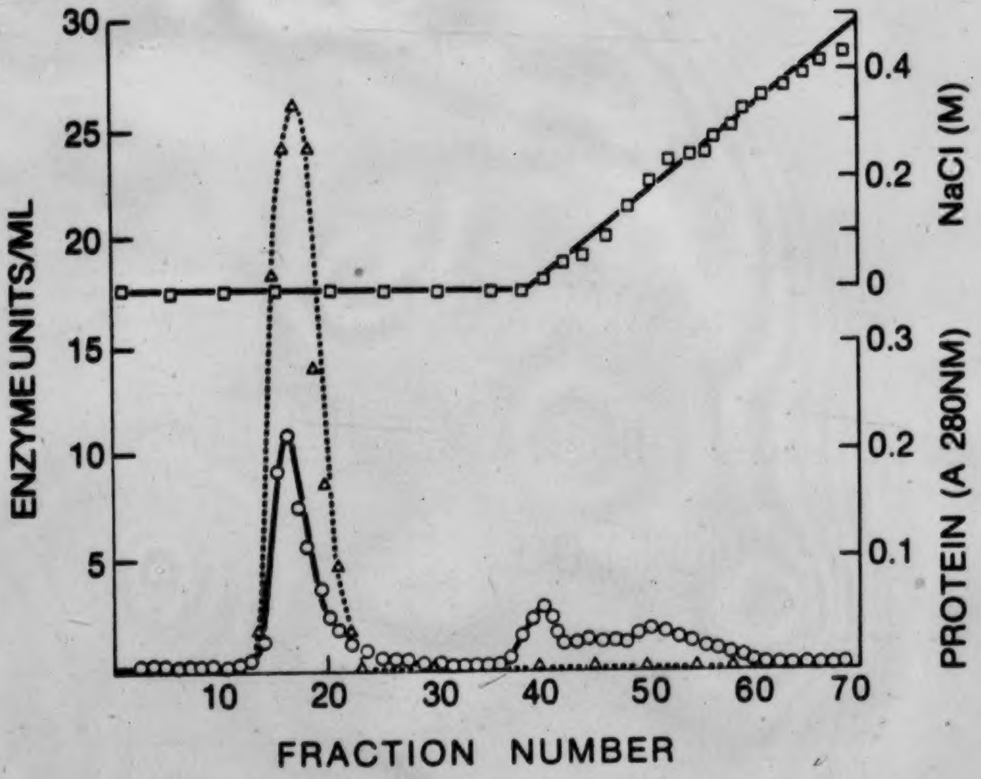
Results are shown in Figure 9, 10, 11, and 12. As can be seen by these results, as the pH increased more protein adsorbed to the column until at pH 9.0, where all the A 280 nm. material and proteolytic activity was bound to the column, requiring 0.03 M NaCl for elution of the latter. When all the figures are compared for proteolytic activity, the most obvious characteristic is that as the pH increased, recovery greatly decreased. For clarity, Table 5 presents a summary of these results.

TABLE 5

EFFECT OF pH ON BINDING OF PROTEASE TO AND ITS ELUTION FROM DEAE-CELLULOSE

pH of Sample[1] and Elution Gradient	Relative Proteolytic Activity (%)	Protein (A 280 nm.) in percent Unbound Fraction	 Bound Fraction
6.0	100	55	45
7.0	87	57	43
8.0	35	36	64
9.0	13	0	100

1. Details on samples and buffers used have been given in text.



30
25
20
15
10
5
ENZYME UNITS/ML
0.4
0.2
0
NaCl (M)
0.3
0.2
0.1
PROTEIN (A 280NM)
10
20
30
40
50
60
70
FRACTION NUMBER

FIGURE 9

ELUTION OF PSEUDOMONAS 461-3-11 PROTEASE FROM DEAE-CELLULOSE (pH 6.0)

An ammonium sulphate precipitate of pH 6.0 (24 mg. protein) was applied to a DEAE-cellulose column. For the size, operation, and elution of the column, see accompanying text. All fractions were 6.8 ml.. The buffer used throughout was 0.01 M NaH_2PO_4-Na_2HPO_4 pH 6.0. Proteolytic activity was determined by the haemoglobin assay, protein by A 280 mm., and concentration of NaCl by conductivity.

Protein	○———○
Proteolytic Activity	△··········△
NaCl	□———□

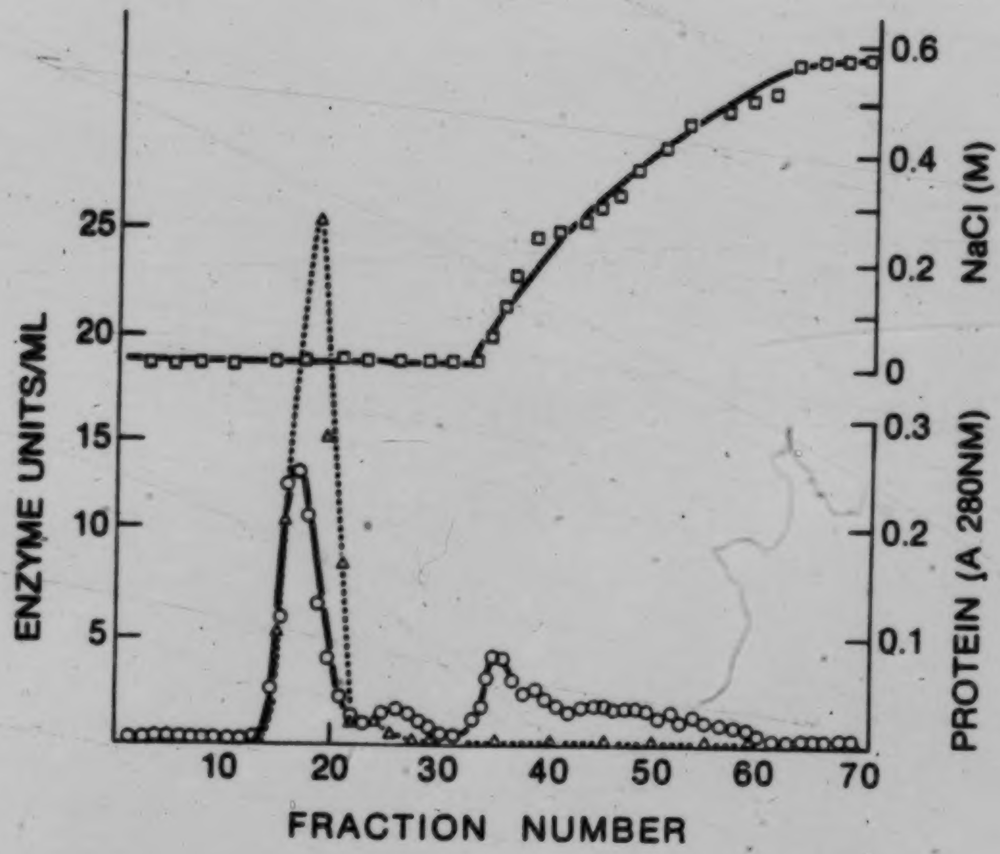

ENZYME UNITS/ML
5
10
15
20
25
0.6
0.4
0.2
0
NaCl (M)
0.3
0.2
0.1
PROTEIN (A 280NM)
10
20
30
40
50
60
70
FRACTION NUMBER

FIGURE 10

ELUTION OF PSEUDOMONAS 461-3-11 PROTEASE FROM DEAE-CELLULOSE (pH 7.0)

All conditions were identical as previously described in Figure 9 except that the buffer was 0.01 M NaH_2PO_4-Na_2HPO_4 pH 7.0.

Protein	○———○
Proteolytic Activity	△··········△
NaCl	□———□

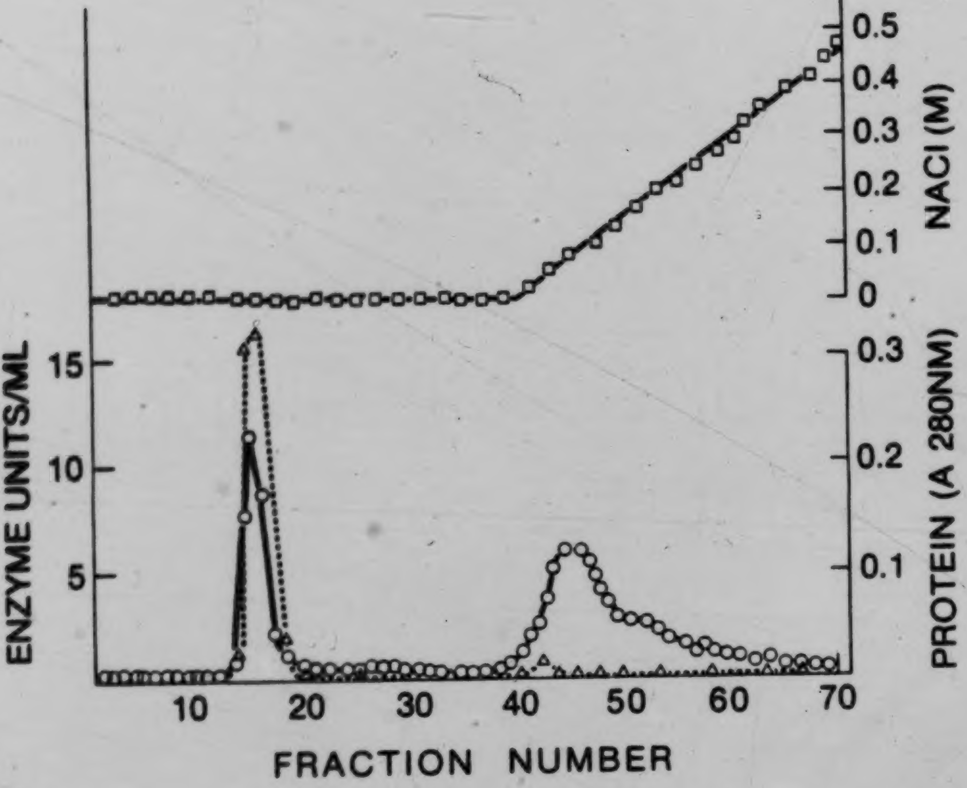

ENZYME UNITS/ML
15
10
5
10
20
30
40
50
60
70
FRACTION NUMBER
0.5
0.4
0.3
0.2
0.1
0
NACI (M)
0.3
0.2
0.1
PROTEIN (A 280NM)

FIGURE 11

ELUTION OF PSEUDOMONAS 461-3-11 PROTEASE FROM DEAE-CELLULOSE (pH 8.0)

All conditions were identical as previously described in Figure 9 except that the buffer was 0.01 M TRIS-HCl pH 8.0.

Protein ○———○

Proteolytic Activity △···········△

NaCl □———□

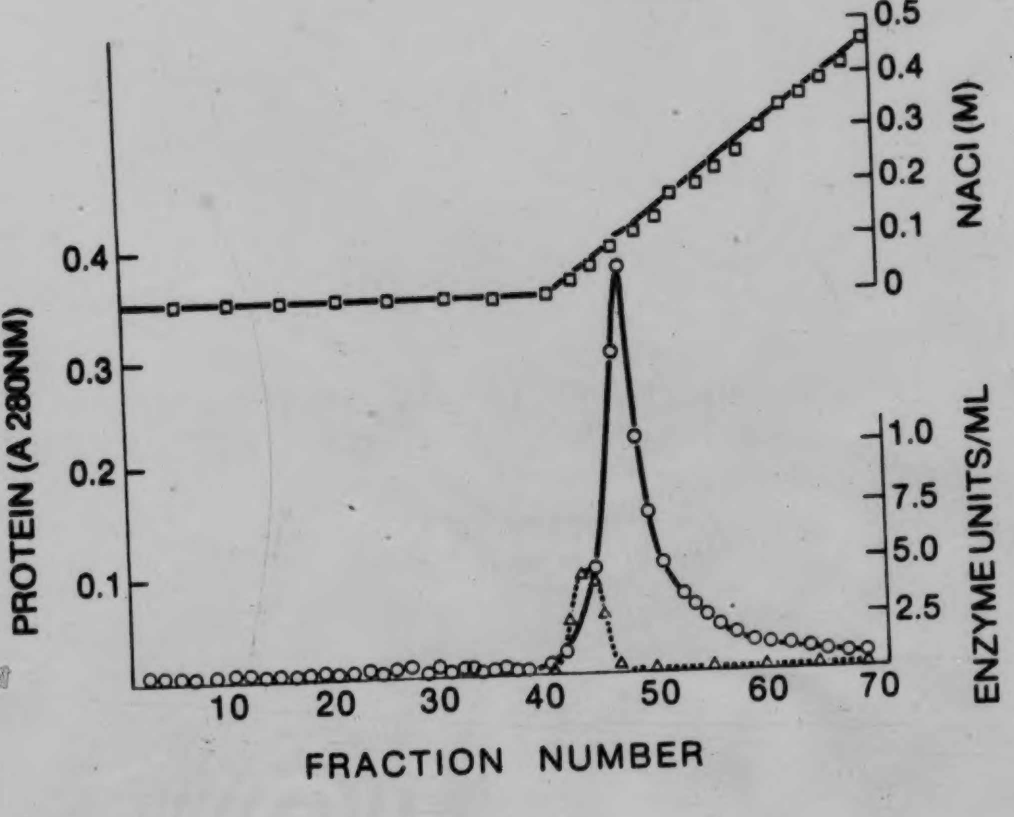

0.4
0.3
0.2
0.1
PROTEIN (A 280NM)
10
20
30
40
50
60
70
FRACTION NUMBER
0.5
0.4
0.3
0.2
0.1
0
NACI (M)
1.0
7.5
5.0
2.5
ENZYME UNITS/ML

FIGURE 12

ELUTION OF PSEUDOMONAS 461-3-11 PROTEASE FROM DEAE-CELLULOSE (pH 9.0)

All conditions were identical as previously described in Figure 9 except that the buffer was 0.01 M TRIS-HCl pH 9.0.

Protein	○——○
Proteolytic Activity	△···········△
NaCl	□——□

Whether the decrease in proteolytic activity was due to pH inactivation or to autodigestion was not investigated. However, the precipition of the enzyme at an acidic pH gave irreproducible results whenever high concentrations of protein were applied. Therefore, pH 8.0 was chosen for purification on DEAE-cellulose. Also, although proteolytic activity was not significantly adsorbed to the column at pH 6.0-8.0 (see Figures 9, 10, and 11), at the latter pH, a larger amount of contaminating protein was removed as is evidenced in Table 4.

One advantage of the use of DEAE-cellulose was the removal of a green pigment that also precipitated in the 36-65% saturation with ammonium sulphate. Although the pigment bound strongly to the column, under alkaline conditions it could be partially eluted with 1.0 M NaCl. Although the pigments of *Pseudomonas spp*. were implicated with pathogenicity, during the early part of the century in Europe, "pyocyanase", an extract from *Pseudomonas aeruginosa*, was actually used to treat infections of anthrax (as quoted by Liu, 1974). Therefore, although DEAE-cellulose proved useful in the partial purification of *Pseudomonas* 461-3-11 proteolytic activity, it may also prove useful in other lines of research such as the purification of pigments or other components within the culture supernatant.

5) CM-cellulose:

The proteolytic peak from the DEAE-cellulose column was concentrated with PEG, then applied to 2.5 cm. X 30 cm. columns of CM-cellulose as described in Materials and Methods. Elution was with a 0-0.3 M NaCl gradient in 0.01 M phosphate buffer pH 6.0 (4°C). The elution profile is shown in Figure 13. As can be seen, three proteolytic peaks were

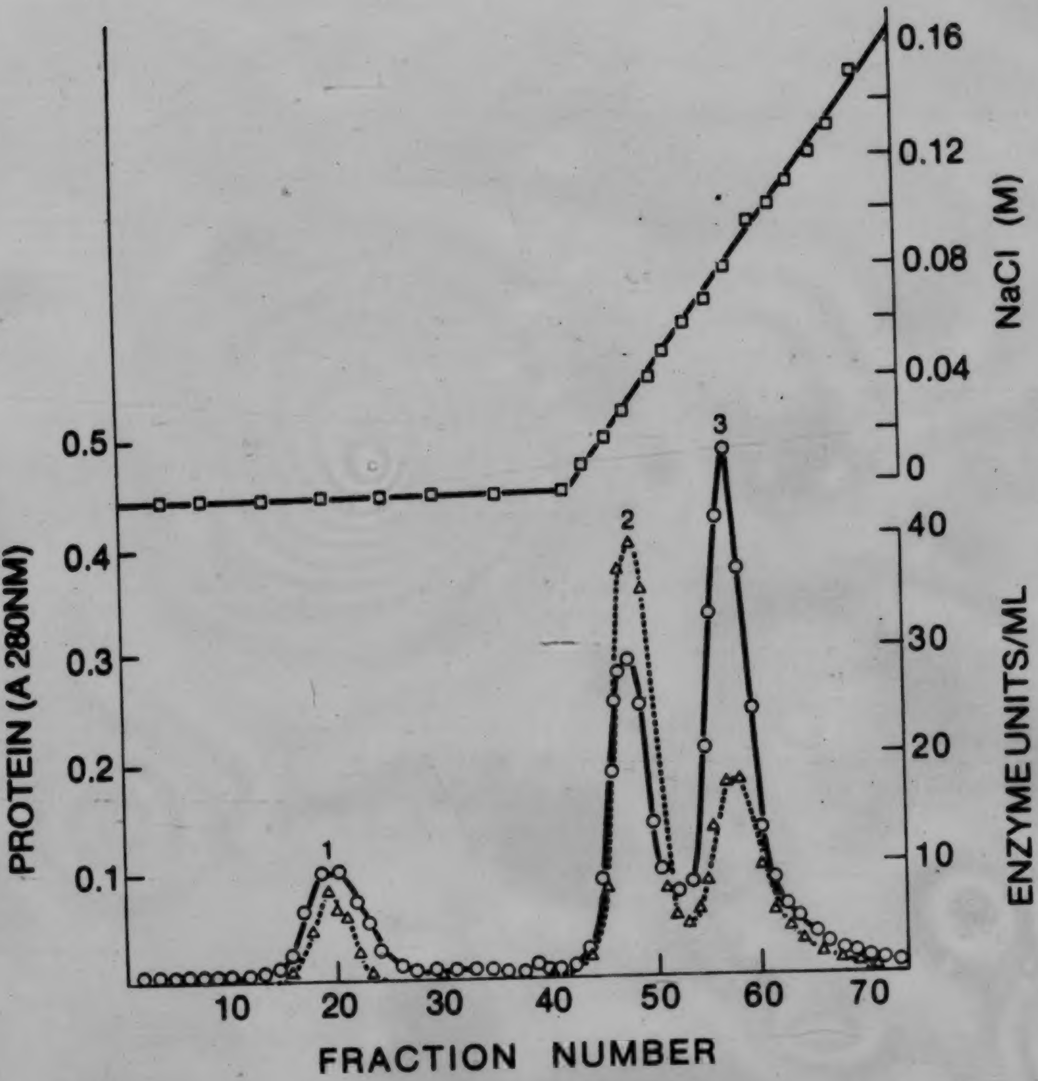

0.5
0.4
0.3
0.2
0.1
PROTEIN (A 280NM)
10
20
30
40
50
60
70
FRACTION NUMBER
0.16
0.12
0.08
0.04
0
NaCl (M)
40
30
20
10
ENZYME UNITS/ML
1
2
3

FIGURE 13

ELUTION PROFILE OF PARTIALLY PURIFIED PSEUDOMONAS 461-3-11 PROTEASE FROM CM-CELLULOSE

The ammonium sulphate precipitate of a 24 hour culture of Pseudomonas 461-3-11 was chromatographed on a DEAE-cellulose column (conditions were as described in Figure 11). The proteolytic peak (36.6 mg. of protein) was then chromatographed on CM-cellulose. For the size, operation, and elution of the latter column, see accompanying text. All fractions were 6.8 ml. and the buffer used was 0.01 M NaH_2PO_4-Na_2HPO_4 pH 6.0. Proteolytic activity was determined by the haemoglobin assay, protein by A 280 nm., and molarity of NaCl by conductivity.

Protein	○——○
Proteolytic Activity	△··········△
Conductivity	□——□

Protease 1 = 1

Protease 2 = 2

Protease 3 = 3

separated, eluting at 0, 0.03, and 0.07 M NaCl in buffer. These were designated Protease 1, 2, and 3 respectively. Equilibrating the column at pH 7.0 or 8.0 (the latter in 0.01 M TRIS-HCl) made no apparent difference in the elution profile.

Within the genus Pseudomonas, having more than one protease appears to be the rule rather than the exception (see Introduction of this text). Whether the proteases of Pseudomonas 461-3-11 bear similarities to the proteases of these or other species was not investigated. Three of the four proteinases elaborated from Streptomyces fradiae (Morihara et al., 1967) and Streptomyces griseus (Wahlby, 1968), for examples, bear striking resemblances to Pseudomonas 461-3-11 proteases both in their elution characteristics and relative amounts isolated on CM-cellulose chromatography.

It has been reported that column dimensions play a key role in the separation of proteins with anion-exchange chromatography. For example, Sabbagh and Fagerson (1976) reported that resolution is a function of the square root of the column's length, while Pitt (1976) reported that separation depended on column length and cross-sectional area. For Pseudomonas 461-3-11 proteases partially purified by DEAE-cellulose, 1.5 cm. X 23 cm. columns of CM-cellulose consistently gave not 3 but 2 protein peaks, one at 0 and another at 0.06 M NaCl either in phosphate buffer pH 6.0 or TRIS-HCl pH 8.0. As these results were from separate experiments under varying conditions, the observation that smaller columns gave poorer resolution than larger columns should be viewed as suggestive rather than conclusive.

6) Disc Gel Electrophoresis:

To determine purity, the three peaks separated by CM-cellulose were subjected to discontinuous polyacrylamide gel electrophoresis. Details are given in the legends accompanying the figures and in Materials and Methods.

i) Polyacrylamide Gels (pH 8.6): Electrophoresis at pH 8.6 yielded the results pictured in Figure 14A. As Protease 1, 2, and 3 were separated by a cation exchanger, it was concluded that by having the cathode as the upper electrode, these enzymes migrated away from the gels. Rather than simply reverse the terminals, the method of Reisfield et al. (1962) was used.

ii) Polyacrylamide gels (pH 4.3): Electrophoresis under these conditions with no pretreatment yielded several inactive bands on each of the gels. This could have been the result of the oxidation of sulphydryl groups of the protein by the ammonium persulphate present in the gel. To test this hypothesis, gels were pre-run for 2 hours to remove residual ammonium persulphate. The results of these experiments are shown in Figure 14B. Several authors have noted that this reagent can cause both inactivation and multiple banding, the latter as a result of differences in oxidation over the population of protein (Mitchell, 1967; Fantes and Furminger, 1967; Bennick, 1968).

For gels 2, 3, and 4, all major bands were active upon extraction with 0.01 M TRIS-HCl buffer pH 7.5. The faint band below the major one on gel 4 was inactive. In a similar manner, Barach et al. (1976) and Yokosawa et al. (1976) have reported success in the use of discontinuous gels at pH 4.3 for their proteases under study.

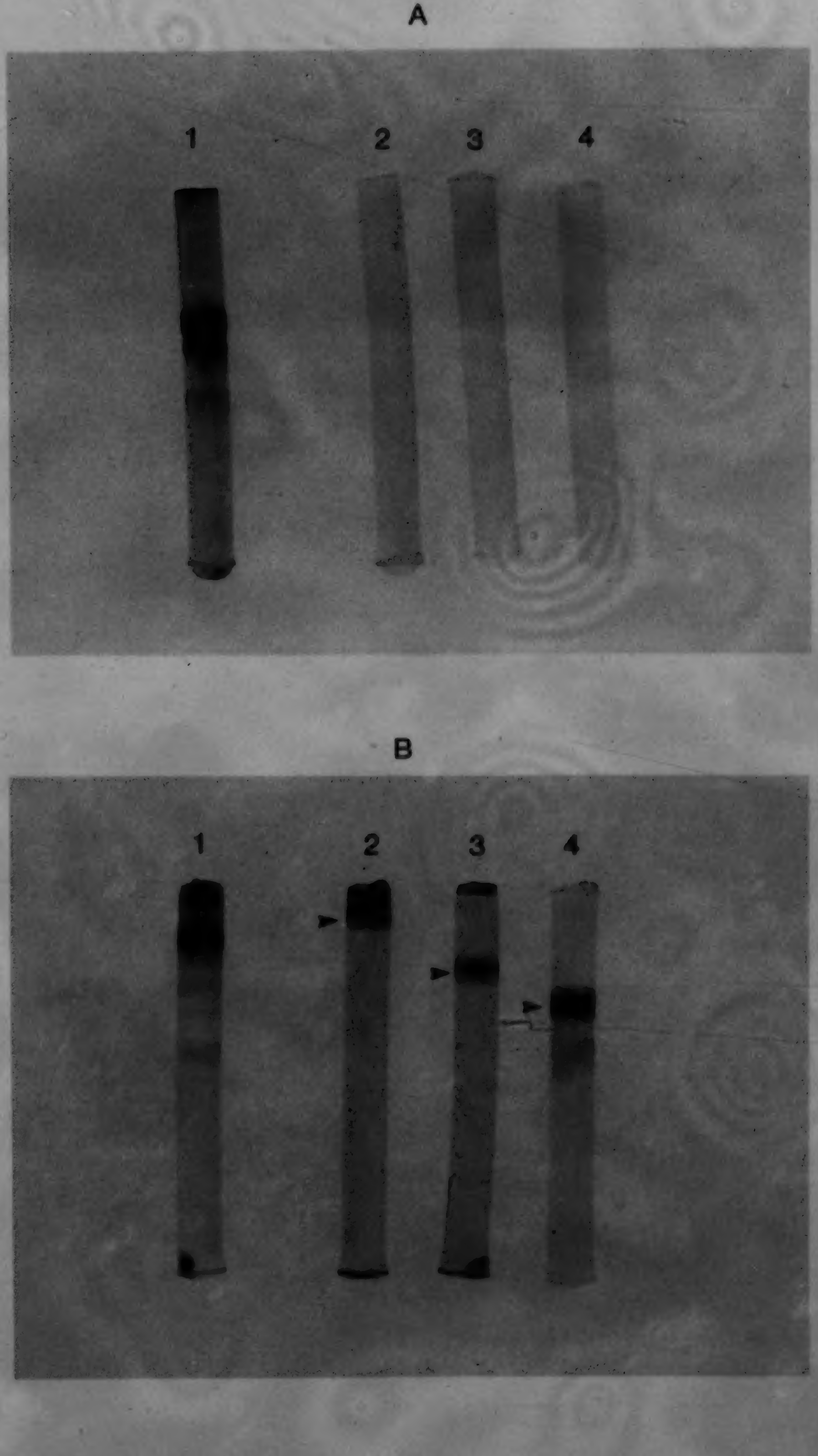

A
1
2
3
4
B
1
2
3
4

FIGURE 14

DISCONTINUOUS POLYACRYLAMIDE GEL ELECTROPHORESIS OF PURIFIED PSEUDOMONAS 461-3-11 PROTEASES

A - pH 8.6

B - pH 4.3

In both cases, preparation of gels, sample application, electrophoresis, and staining of gels are described in Materials and Methods. Stacking gels have been removed.

Gel Number	Sample
1	100 μgm. crude supernatant of culture
2	50 μgm. Peak 1 from Figure 13
3	50 μgm. Peak 2 from Figure 13
4	50 μgm. Peak 3 from Figure 13

The position of active bands have been noted by: ►

7) Summary:

For Table 6, fifteen 2 litre flasks, each with 200 ml. of 3% TCS, were inoculated and then incubated at 22°C under 300 rpm. agitation for 36 hours. The purification sequence for the Pseudomonas 461-3-11 proteases followed that noted at the beginning of this section. The CM-cellulose step has not been included in a comparison with the other techniques, because the total enzyme units and protein did not differ significantly from Peak 1 of the DEAE-cellulose step. There was a 28 fold purification of proteolytic activity from the crude supernatant of the culture to the CM-cellulose step.

8) Unsuccessful Procedures Attempted:

i) Ultrafiltration: For large volumes of crude supernatant from cultures of Pseudomonas 461-3-11, ultrafiltration proved cumbersome and lengthy. Initial concentration was done on Model TC1C for 4 days, which had a lower limit of 1 litre. This volume was concentrated further on Model 400 (Amicon Corporation) for 1-2 days. Aside from blockage of the membranes, recovery of proteolytic activity by this series of filtrations was determined to be 42%. Precipitation of the protease by ammonium sulphate proved more convenient.

ii) Gel Exclusion Chromatography: A 2.5 cm. X 38 cm. column of Sephadex G-100 was found effective for fractionating 30 mg. of Pseudomonas 461-3-11 crude supernatant concentrated by ultrafiltration. However, when large amounts of protein were applied to similar columns (e.g. 1.05 grams was attempted), poor resolution occurred as a result of overloading. Cellulose chromatography was found to be less restrictive.

iii) Isoelectric Focusing: Purified Protease 2 which gave a single

TABLE 6

TYPICAL PURIFICATION OF PSEUDOMONAS 461-3-11 PROTEASES

Sample	Total Enzyme Units	Recovery (%)	Total Protein (grams)	Recovery (%)	Specific Activity (eu./mg. protein)
Sonicate of Culture	54,000	100	19.2	100	2.8
Supernatant of Culture	58,000	107	12.1	63	4.8
Ammonium Sulphate Fractionation (36-65% sat.)	29,000	54	1.31	6.8	22.1
Peak 1 of DEAE-cellulose	26,300	40	0.195	1.0	134.9

Proteolytic activity was determined by the casein assay.

band on polyacrylamide gels (pH 4.3), upon application to the IEF apparatus gave inconsistent results with much loss of activity. It has been reported in some cases that ampholytes will chelate and remove essential metals (Righetti and Drysdale, 1974). Boethling (1975) reported that during the purification of a protease from Pseudomonas maltophilia there was a loss of activity and irreproducible results due to the extraction of calcium metals by the IEF apparatus. This procedure was therefore abandoned.

III) CHARACTERIZATION OF PSEUDOMONAS 461-3-11 PROTEASES

1) Localization of Activity Within and Without the Cell:

For Table 7, 300 ml. of 3% TCS in a 2 litre flask was inoculated, incubated at 23°C with agitation at 300 rpm., and then had two 3 ml. aliquots removed at noted times. Only one of these samples had its cells removed by centrifugation at 27,000 X g for 30 minutes. All samples were diluted 1:10 in distilled water and were disrupted with the same oscillation as described in Materials and Methods. The efficiency of cell breakage was estimated visually to be 90%. Proteolytic activity was determined by the azocoll assay.

As can be seen from Table 7, all four samples had approximately the same proteolytic activity, indicating that essentially none of the detectable protease is cell-associated. Cell breakage did not cause the levels of this enzyme to increase significantly. These results are similar to those of May and Elliott (1968) who reported that the extracellular protease of Bacillus subtilis required de novo protein synthesis and that in no instance was a pool of enzyme present within the cell.

Although the samples had approximately the same proteolytic activity, of the four, sonication of the crude culture having whole cells initially gave the lowest readings. As there are several proteases within the cell (Miller, 1975; Pacaud and Uriel, 1971), one would expect the highest, not the lowest proteolytic activities within these upon cell disruption. Two possibilities that might explain this discrepancy are the release of internal salts into the medium upon breakage (see subsection 4 dealing with osmotic sensitivity of the enzymes) or the presence of an inhibitor within the cell as a guard against back-diffusion (Smeaton

TABLE 7

EFFECT OF CELL DISRUPTION ON THE LEVEL OF PROTEOLYTIC ACTIVITY FOR A CULTURE OF PSEUDOMONAS 461-3-11

Sampling Time (Hrs.)	OD 600 nm. of Culture	Proteolytic Activity (eu./ml.)			
		Culture	Sonicated Culture	Supernatant	Sonicated Supernatant
0	.03	0	0	0	0
8.5	6.09	1.38	0.56	0.58	0.76
10.5	9.81	1.76	1.14	1.20	0.98
12.5	11.28	2.94	2.12	2.80	2.94
14.5	12.31	9.66	8.26	9.36	9.24
16.5	12.67	18.4	15.2	18.2	17.7
22.5	14.34	23.6	20.8	22.1	21.7

Proteolytic activity was determined by the Azocoll Assay.

Sonication was 12 times at 15 seconds per time at 20,000 cps.

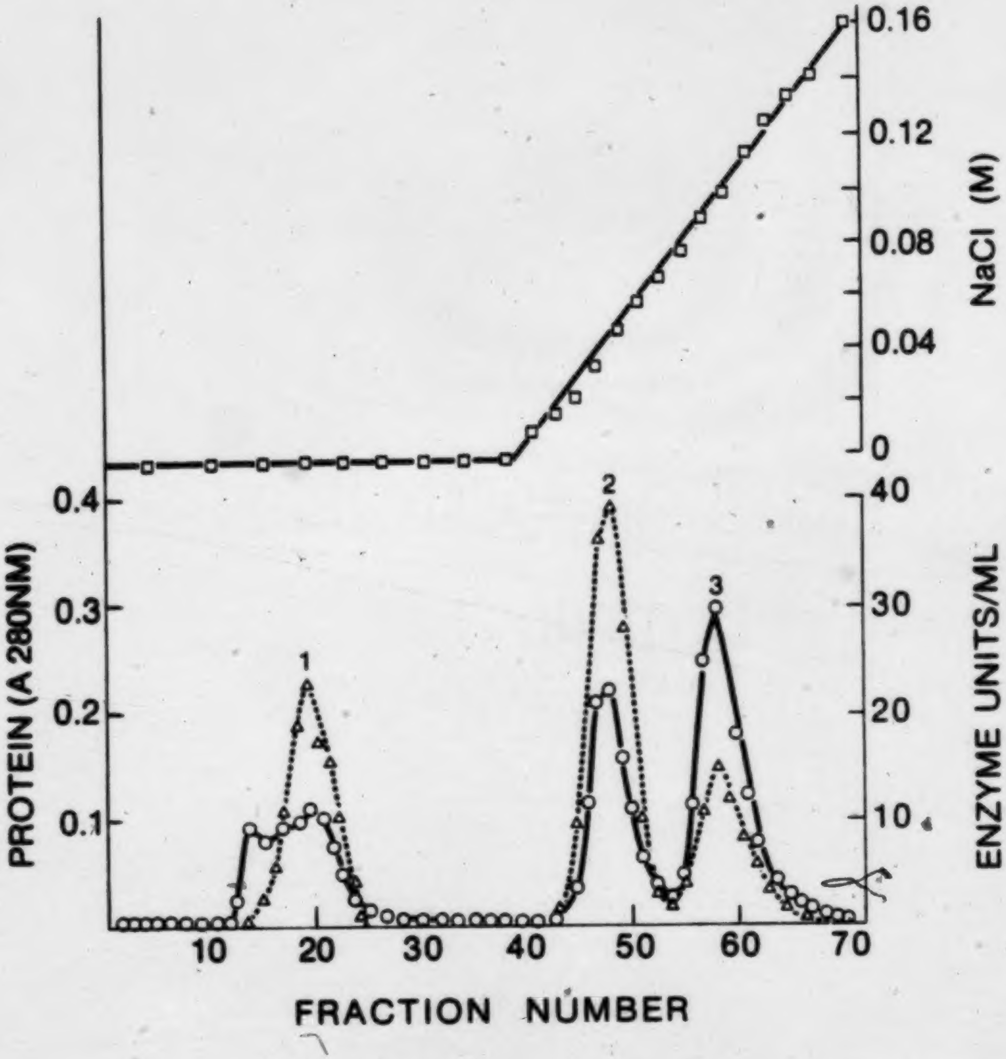
and Elliott, 1967; Hartley, 1970).

2) Stability:

As enzymes are proteins, a common problem in the purification of proteases is loss due to autodigestion. Figures 13, 15, and 16 show the elution profiles from CM-cellulose obtained with 3 identical samples under similar conditions with the exception that preincubations differed prior to elution through CM-cellulose. Figure 13 shows the result of an elution immediately after the DEAE-cellulose step, Figure 15 had the second sample preincubated 14 days at 4°C, and Figure 16 had the third sample at 22°C for 10 days. It can be seen that the A 280 nm. material and proteolytic activity for peaks 2 and 3 decreased while in peak 1 these properties increased as the preincubation period increased. Electrophoresis of this latter peak was not undertaken.

Even storage at -20°C was not sufficient to prevent degradation of the purified proteases. Figures 17A and 17B show the results obtained when purified Proteases 1, 2, and 3 of Pseudomonas 461-3-11 were left for 3 months at this temperature and then analyzed electrophoretically. If one compares these with Figures 14A and 14B in which "fresh" enzymes were subjected to electrophoresis, the appearance of new bands upon aging is evident. Of the three enzymes, Protease 3 appears to be the most stable as new bands are not apparent either in gels of pH 8.6 or of pH 4.3. Time permitted only the analysis of the latter gels for proteolytic activity. All bands were found active except for the minor band below Protease 3.

Loss of enzyme activity in purified preparations of proteases has been reported in a Sarcina strain (Coccus P) by Bissell et al. (1971)



0.16
0.12
0.08
0.04
0
NaCl (M)
2
3
1
40
30
20
10
0
ENZYME UNITS/ML
0.4
0.3
0.2
0.1
PROTEIN (A 280NM)
10
20
30
40
50
60
70
FRACTION NUMBER

FIGURE 15

ELUTION PROFILES OF PSEUDOMONAS 461-3-11 PROTEASES FROM CM-CELLULOSE AFTER PREINCUBATION 14 DAYS AT 4°C

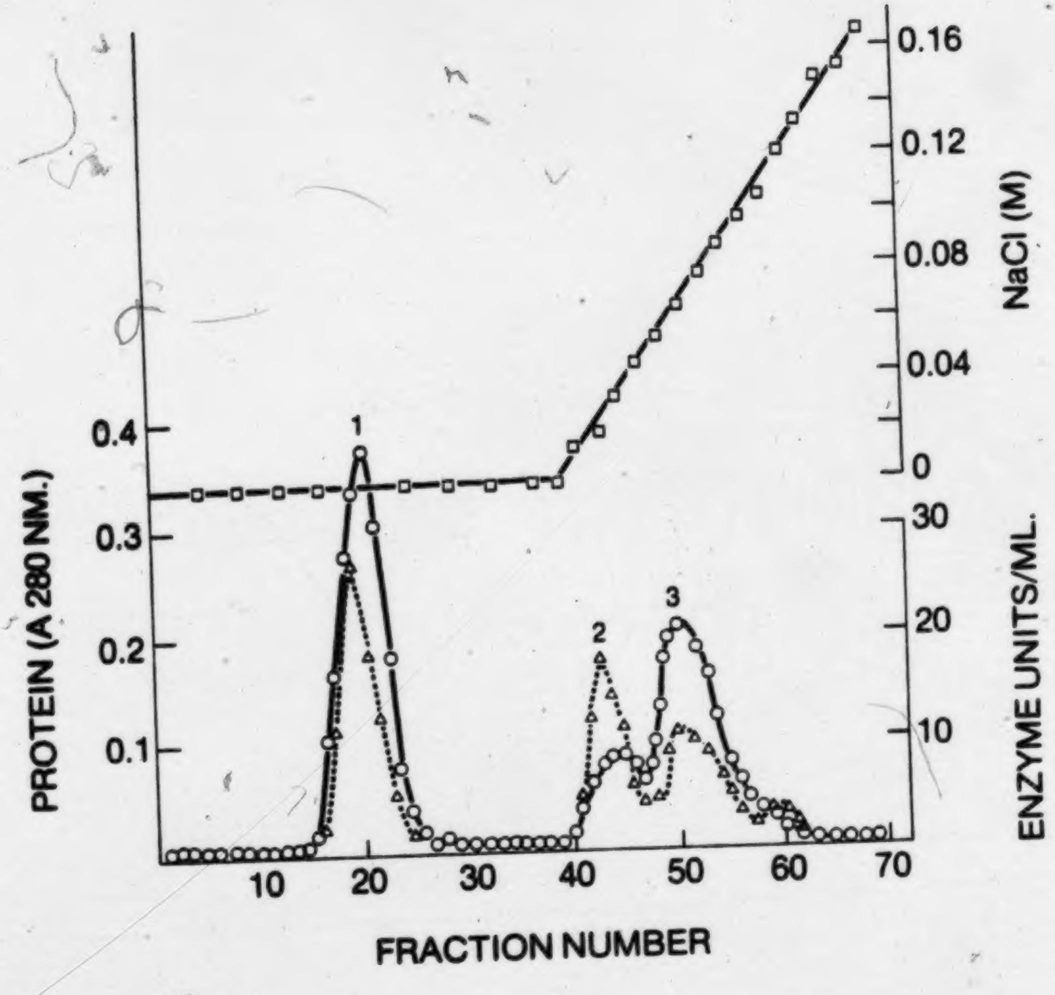
The protease peak from the DEAE-cellulose column (2.8 mg. protein per ml., 13 ml. total volume) was incubated in the presence of 0.05% sodium azide for 14 days at 4°C. After this time it was chromatographed on CM-cellulose as described in the legend of Figure 13.

Protein	○———○
Proteolytic Activity	△··········△
NaCl	□———□

Protease 1 = 1

Protease 2 = 2

Protease 3 = 3



1
2
3
PROTEIN (A 280 NM.)
0.4
0.3
0.2
0.1
NaCl (M)
0.16
0.12
0.08
0.04
0
ENZYME UNITS/ML.
30
20
10
10
20
30
40
50
60
70
FRACTION NUMBER

FIGURE 16

ELUTION PROFILE OF PSEUDOMONAS 461-3-11 PROTEASES FROM CM-CELLULOSE AFTER PREINCUBATION 10 DAYS AT 22°C

All conditions were identical to Figure 13 except that the sample was preincubated 10 days at 22°C.

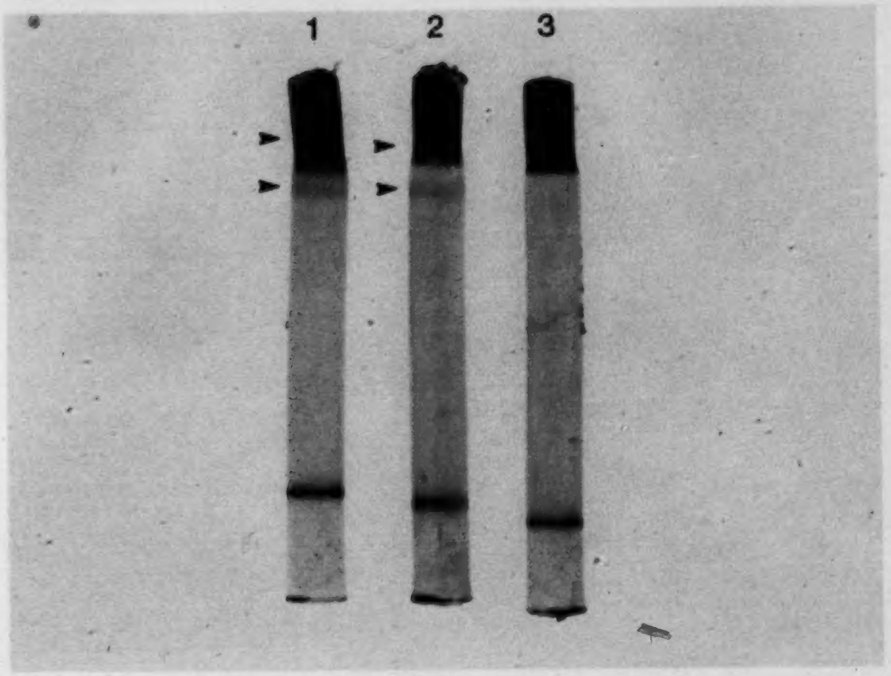
Protein	○———○
Proteolytic Activity	△··············△
NaCl	□———□

Protease 1 = 1

Protease 2 = 2

Protease 3 = 3

A



B

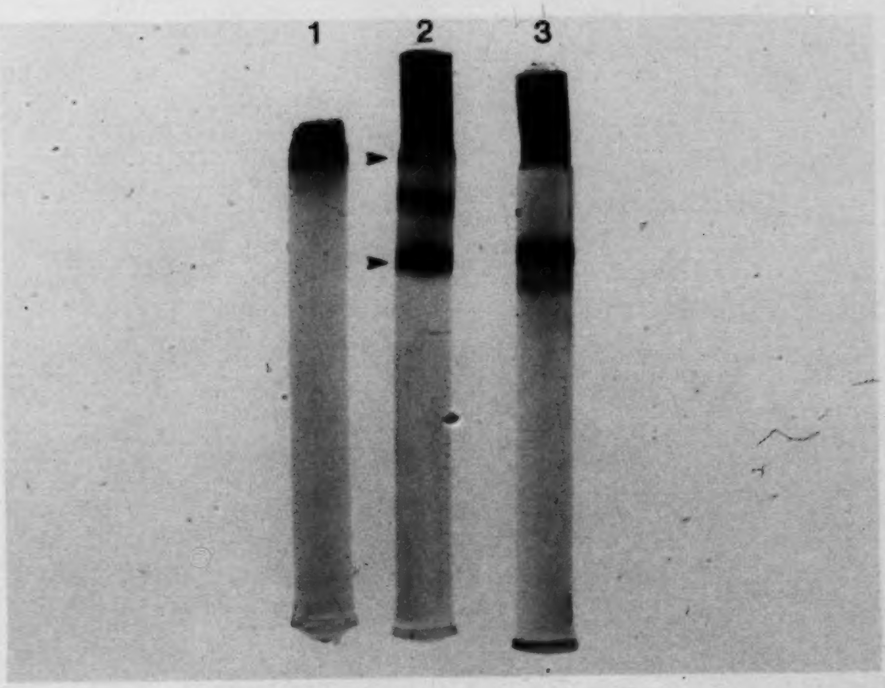


FIGURE 17

DISC GEL ELECTROPHORESIS OF PURIFIED PSEUDOMONAS
461-3-11 PROTEASES STORED AT -20°C FOR 3 MONTHS
A - pH 8.6
B - pH 4.3

The separate protein peaks shown in Figure 14, after storage at -20°C for 3 months, were run on polyacrylamide gels.

Polyacrylamide Gel	Sample
1	50 μgm. Protease 1
2	50 μgm. Protease 2
3	50 μgm. Protease 3

The position of new bands are noted by the symbol ►

and in a Serratia species by Miyata et al. (1970a). Sarner et al. (1971) quantitated the autodigestion of a Sarcina strain (Coccus P) by incorporating ^{14}C-leucine into the enzyme, then noting the rise in radioactivity in the TCA soluble fraction. Although time did not permit such an analysis, the experiments dealing with change in behaviour on ion-exchange chromatography and polyacrylamide gels suggests that autodigestion of Pseudomonas 461-3-11 proteases does occur.

3) Collagenolytic Activity:

As Pseudomonas 461-3-11 was discovered and isolated because of its action on steerhides, the hydrolysis of this largely insoluble material by purified proteases was investigated. Collagenolytic activity was determined by the procedure noted in Materials and Methods.

Of the three enzymes, only Protease 3 exhibited an ability to solubilize collagen. Unlike "true collagenases" which can hydrolyze the helical regions of collagen (Mandl, 1963), Pseudomonas 461-3-11 acts against the non-helical regions (Giffee et al., 1965; Giffee, 1961), causing loss of covalent interpeptide bonds and hence dispersal upon denaturation. Other species of Pseudomonas have been reported to have collagenolytic activity (Schoellman and Fisher, 1966; Adamcic and Clark, 1970). Kucera and Lysenko (1968), for example, have isolated 5 proteases from Pseudomonas aeruginosa varying in their properties. All but enzyme A were found to have collagenase activity while all but enzyme D had elastinase activity.

4) Sensitivity to External Agents:

i) Ionic Strength: Preliminary experiments, using high concentrations of buffers to determine pH optima, led to the conclusion that the 3 enzymes of Pseudomonas 461-3-11 were extremely sensitive to the molarity of solutes. To test the sensitivity of the crude protease to ionic strength, the following experiment was arranged. 0.05 ml. aliquots of a supernatant of a 24 hour culture of Pseudomonas 461-3-11 grown on TCS were assayed for protease activity by the azocoll assay in the presence of increasing amounts (0-2.0 M) of:

a) TRIS-HCl buffer pH 7.5
b) NaCl (in 0.01 M TRIS-HCl pH 7.5)
c) Sucrose (in 0.01 M TRIS-HCl pH 7.5)

In this experiment, preincubation times, pH, temperature, and substrate concentrations were constant. The only introduced variable was the given molarities of solutes within the buffer. No studies were done on the sensitivities of the individual proteases.

As shown in Figure 18, the greater sensitivity to TRIS or NaCl than to sucrose indicate that the restriction of the amount of available water present is not the sole explanation. Of particular significance is the slight stimulation by 0.02 M TRIS followed by a rapid decrease in activity as the ionic strength is increased. Although the mechanism has not been clarified, a similar result has been reported by Burke and Pattee (1967) for a Pseudomonas aeruginosa enzyme that lyses a species of Staphylococcus. The sensitivity of proteases to their ionic environment has been noted in several papers. The research of Morihara et al. (1965) on Pseudomonas aeruginosa elastinase and of Setlow (1976) on a Bacillus megaterium spore protease have both yielded enzymes that are inactivated by molar NaCl while Carrick and Berk (1975) have reported that a Pseudomonas aeruginosa collagenase is six times more active in

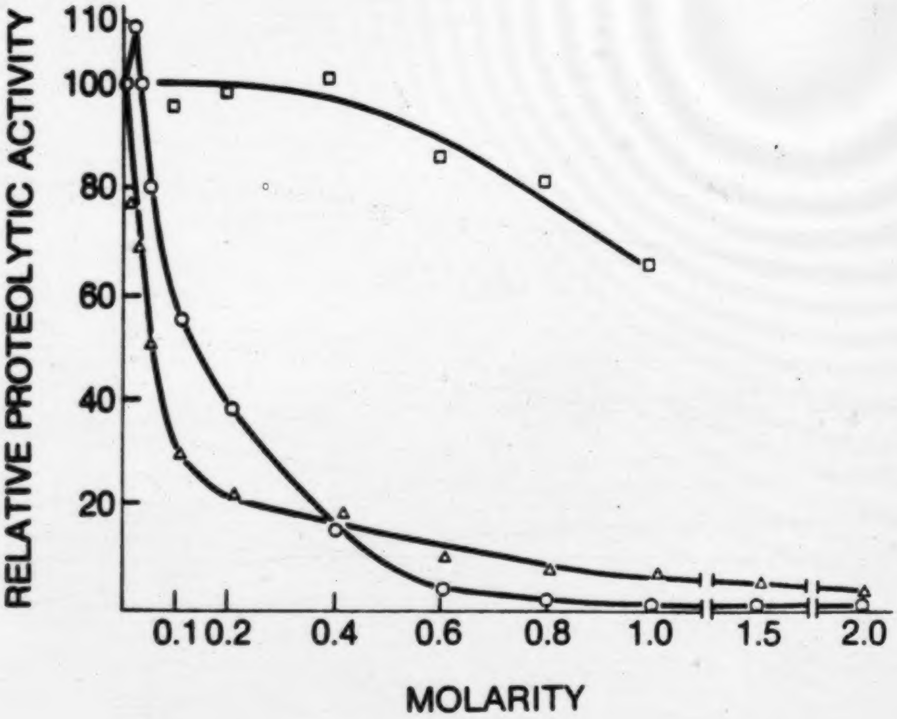

RELATIVE PROTEOLYTIC ACTIVITY
110
100
80
60
40
20
0.1
0.2
0.4
0.6
0.8
1.0
1.5
2.0
MOLARITY

FIGURE 18

THE EFFECT OF MOLARITY OF DIFFERENT COMPOUNDS ON THE PROTEOLYTIC ACTIVITY OF A PSEUDOMONAS 461-3-11 CULTURE

A culture supernatant of a 24 hour TCS grown culture was assayed for proteolytic activity by the azocoll assay under the following solute conditions. Activity of crude supernatant in the standard assay (25 mg. azocoll in 10 ml. 0.01 M TRIS-HCl pH 7.5) was assigned a value of 100.

TRIS-HCl pH 7.5	○——○
NaCl in 0.01 M TRIS-HCl pH 7.5	△——△
Sucrose in 0.01 M TRIS-HCl pH 7.5	□——□

0.1 M TRIS buffer pH 7.5 than 0.1 M phosphate buffer pH 7.5.

ii) pH: Five μgm. of the individual purified proteases in 0.05 ml. of distilled water were added to reaction mixtures of the casein assay. The results are as presented in Figure 19 (for further experimental details, refer to the legend of this figure). The pH optima for Proteases 1, 2, and 3 were found to be 9.3, 9.5, and 8.5 respectively. In comparison with other organisms, aside from protease 1 of Pseudomonas aeruginosa which has a pH optimum of 6.5 (Morihara, 1964), most proteases isolated from Gram-negative bacteria have been found to have an alkaline pH optimum (Miyata et al., 1970b; Keen et al., 1967; Morihara et al., 1965). Also, for Pseudomonas 461-3-11, it should be noted that during growth in TCS, the pH of the culture medium will rise, to a maximum of 8.6-9.2. In an environment that becomes strongly alkaline, extracellular proteases exhibiting alkaline pH optima should come as no great surprise.

iii) Chelating Agents: In the characterization of proteases, one group has been classed by virtue of its sensitivity to metal-chelators. To determine if the proteases of Pseudomonas 461-3-11 were within this classification, the azocoll assay was used with two modifications. The first was that various concentrations of EDTA (adjusted to pH 7.5 with NaOH) were diluted in 0.01 M TRIS-HCl. The second was the preincubation of the enzyme for 15 minutes at 37°C in the TRIS:EDTA solutions before the addition of the substrate. Relative, rather than absolute trends should be considered as the reagents may contribute trace metals to the solution (Morihara, 1974).

Table 8 demonstrates that all three enzymes were inactivated by EDTA although Protease 3 is clearly more resistant than either Protease 1 or 2. The significance of these differences will be elaborated in

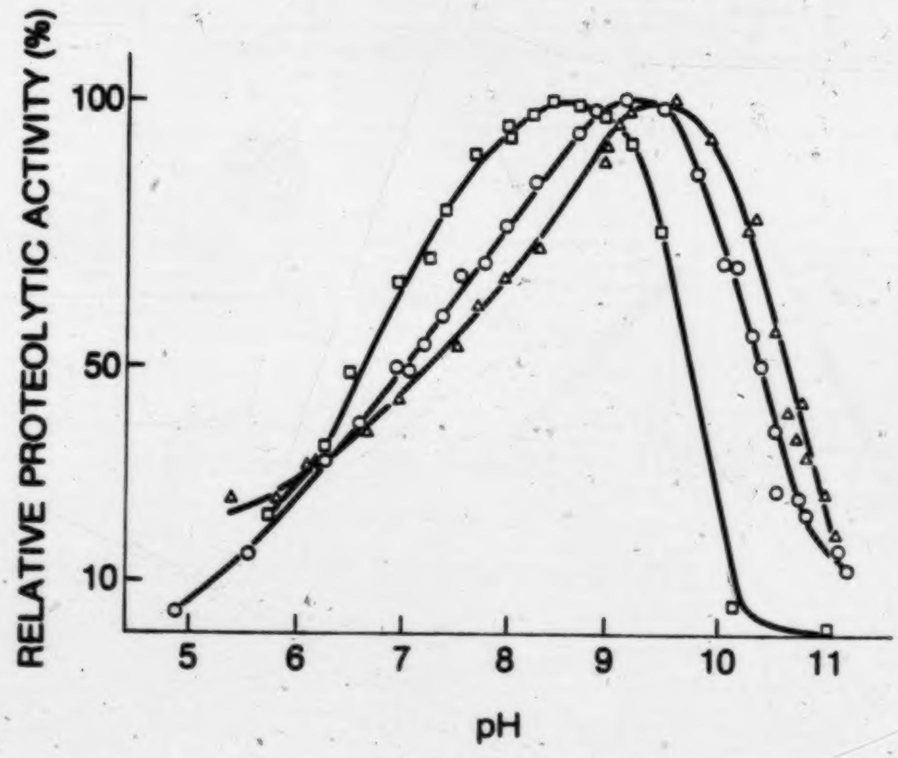

RELATIVE PROTEOLYTIC ACTIVITY (%)
100
50
10
5
6
7
8
9
10
11
pH

FIGURE 19

RELATIVE ACTIVITY OF PSEUDOMONAS 461-3-11 PURIFIED PROTEASES 1, 2, AND 3 AT DIFFERENT pH

The proteases were purified by the method described in the text. Incubation mixtures were: 1 ml. of soluble casein in 0.02 M TRIS; 0.02 M NaH_2PO_4, 0.02 M HCl, or 0.02 M NaOH (used either solely or in combination to adjust the pH); and 0.05 ml. of the purified enzyme in distilled water. 0.02 M NaCl was used to bring the final volume to 3.0 ml.

Protease 1 ○———○

Protease 2 △———△

Protease 3 □———□

TABLE 8

INACTIVATION OF *PSEUDOMONAS* 461-3-11 PROTEASES BY EDTA

EDTA (Molarity)	Relative Activity (%)		
	Protease 1	Protease 2	Protease 3
0	100	100	100
5 X 10^{-7}	93	93	96
1 X 10^{-6}	79	47	102
2 X 10^{-6}	43	95	93
4 X 10^{-6}	1	1	65
6 X 10^{-6}	1	1	58
8 X 10^{-6}	1	1	63
1 X 10^{-5}	2	3	54
2 X 10^{-5}	1	1	48
4 X 10^{-5}	0	0	30
6 X 10^{-5}	0	0	20
8 X 10^{-5}	0	0	15
1 X 10^{-4}	0	0	8
5 X 10^{-4}	0	0	5
4 X 10^{-3}	0	0	1
2 X 10^{-2}	0	0	0
1 X 10^{-1}	0	0	0

The Azocoll Assay was used for the above. All enzyme samples used were 5 µgm. of protein as determined by the method of Lowry *et al.* (1951).

the summary of this section. Time did not permit either studies with other chelators (e.g. EGTA, o-phenanthroline, and 8-hydroxyquinoline) or of the mechanism of EDTA inhibition.

5) Molecular Weight:

To determine the molecular weight of the three purified enzymes of *Pseudomonas* 461-3-11, 50 µgm. of each of proteases 1, 2, and 3, as well as standard proteins of known molecular weight (see legend to Figure 20) were denatured and analyzed electrophoretically. The slab-gel electrophoresis followed the procedure of Ames (1974), the reagents listed in Materials and Methods were used as according to the procedure of Laemmli (1970).

Figure 20 is a sketch illustrating the separation of various polypeptides obtained. The molecular weights for all but haemoglobin are as noted by Dunker and Rueckert (1969). For this protein, although the compound is a tetramer with a total molecular weight of 64,000, under 5% 2-mercaptoethanol it will dissociate to dimer and finally to monomer subunits (Tyuma *et al.*, 1966). Trypsin shows several faint bands of lower molecular weight than 24,000, probably as a result of autodigestion. Also, although lysozyme has a larger molecular weight than ribonuclease A, the former has migrated further, possibly due to abnormal SDS binding (Weber *et al.*, 1972). Protease 1 and 2 were unusual in that instead of one band, several were present in the same proximity. Whether this was due to autodigestion, abnormal SDS binding, or several subunits of the enzymes having similar molecular weight was not resolved.

Following the method of Dunker and Rueckert (1969), Figure 21

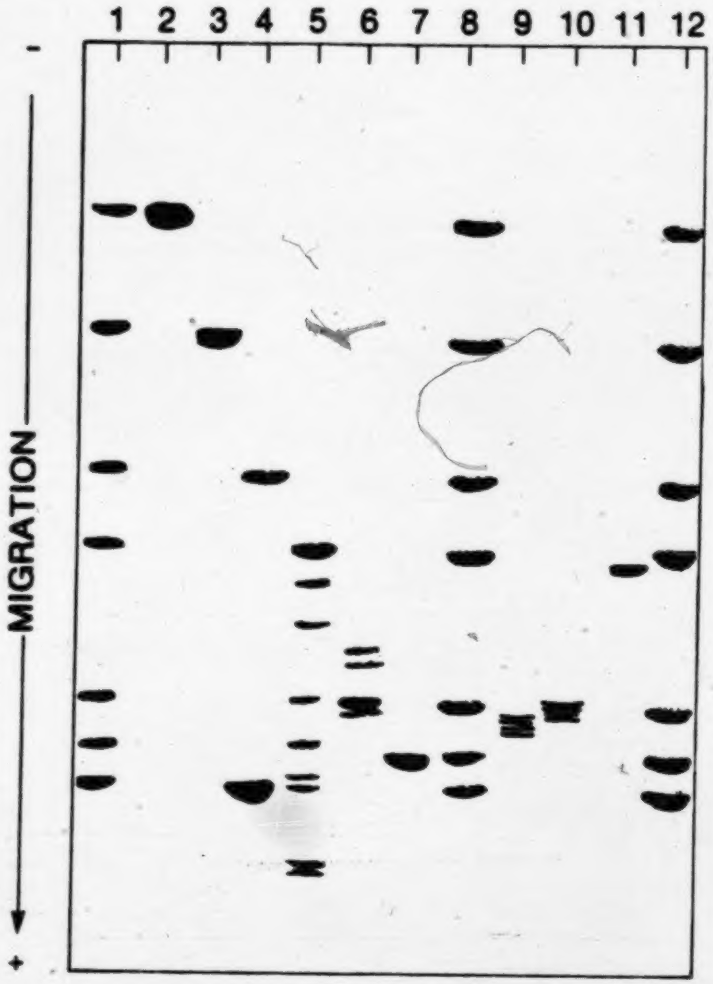

-
MIGRATION
+

FIGURE 20

SEPARATION OF POLYPEPTIDES BY SDS-GEL ELECTROPHORESIS

50 μgm. each of purified Pseudomonas 461-3-11 proteases 1, 2, and 3 and the designated control proteins were analyzed electrophoretically as described in Materials and Methods.

Slot Number	Sample	Molecular Weight
1	Mixture of Protein Standards	
2	Bovine Serum Albumin	66,000
3	Ovalbumin	46,000
4	Haemoglobin: dimer unit	32,000
	monomer unit	16,000
5	Trypsin	23,800
6	Ribonuclease-A	13,680
7	Lysozyme	14,400
8	Mixture of Protein Standards	
9	Protease 1	
10	Protease 2	
11	Protease 3	
12	Mixture of Protein Standards	

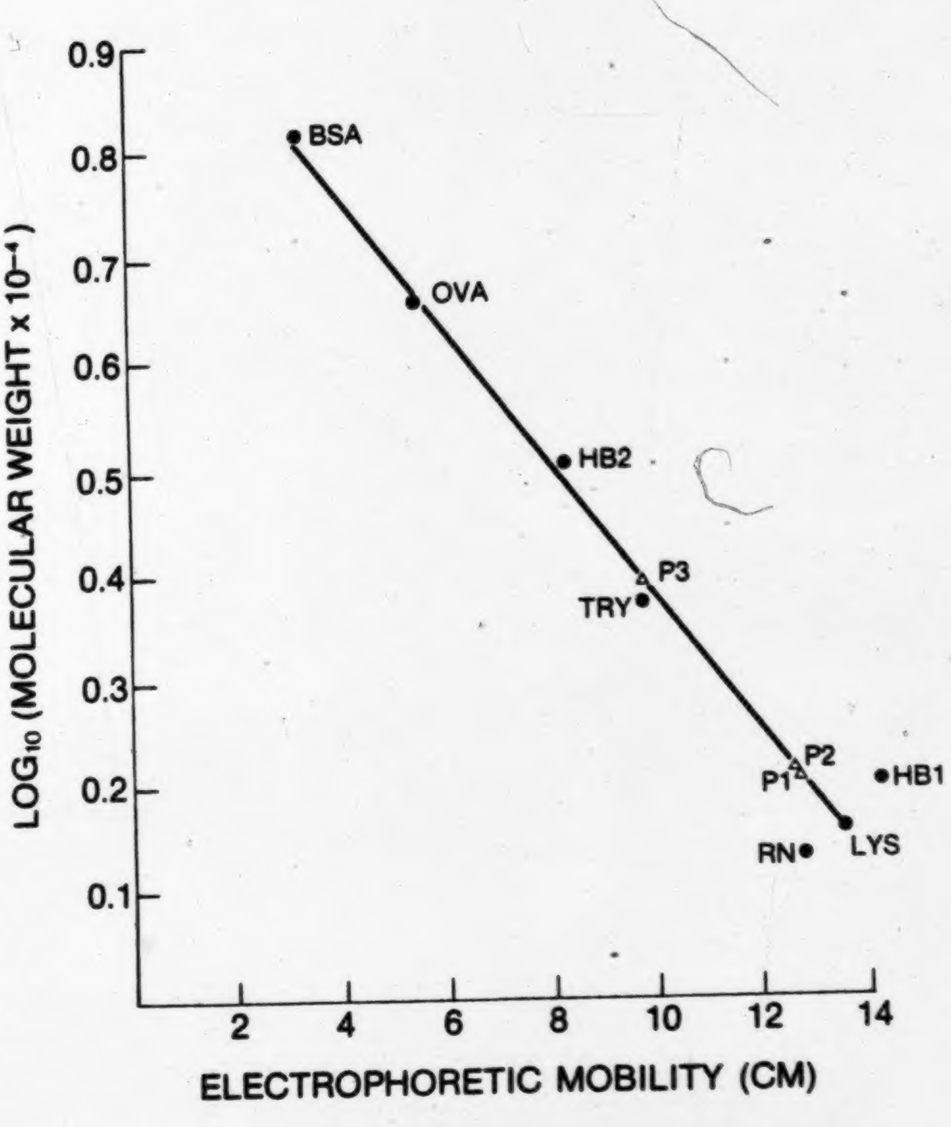

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
LOG10 (MOLECULAR WEIGHT x 10⁻⁴)
BSA
OVA
HB2
P3
TRY
P2
P1
HB1
LYS
RN
2
4
6
8
10
12
14
ELECTROPHORETIC MOBILITY (CM)

FIGURE 21

COMPARISON OF MOLECULAR WEIGHTS OF POLYPEPTIDES SEPARATED BY SDS-GEL ELECTROPHORESIS

The data from Figure 20 has been plotted as shown.

BSA - Bovine Serum Albumin
OVA - Ovalbumin
HB2 - Haemoglobin (dimer)
HB1 - Haemoglobin (monomer)
TRY - Trypsin
LYS - Lysozyme
RN - Ribonuclease-A
P1 - *Pseudomonas* 461-3-11 Protease 1
P2 - *Pseudomonas* 461-3-11 Protease 2
P3 - *Pseudomonas* 461-3-11 Protease 3

presents the above data in graphic form. From the interpolation of these results, it was estimated that the molecular weights of Proteases 1, 2, and 3 were 31,000, 33,000, and 59,000 respectively. Whether these were homologous enzymes or subunits of similar molecular weights dissociated by 2-mercaptoethanol was not determined.

6) Summary:

Table 9 presents a summary of the characterization of Pseudomonas 461-3-11 proteases 1, 2, and 3. Although several topics have been undertaken, in two ways the characterization is deficient. The first, as with most characterizations, is that by no means is it complete. Sedimentation coefficients, amino acid analysis, pH or temperature stability, carbohydrate or lipid content, and sites of substrate cleavage are all basic studies that were not done on the three enzymes. The second is that superficial rather than in depth investigations have been done. The enzymes were found to be inactivated by EDTA. What was the mechanism of this inhibition? Had EDTA chelated a metal essential for activity or stability? If so, what were these metals? What are the effects of other chelators? Did pH, ionic strength, or temperature influence this inhibition?

However, just as there are deficiencies, so the data also present insight and possible directions of research. All three enzymes neither adsorbed to DEAE-cellulose nor showed anionic mobility under alkaline conditions (i.e. pH 8.0 for the former and pH 8.6 for the latter). Does the cationic nature of the freshly purified enzyme play a role in activity or substrate specificity? The molecular weights, EDTA sensitivities, pH optima, and collagenolytic activities presented in

TABLE 9

SUMMARY OF THE CHARACTERIZATION OF *PSEUDOMONAS* 461-3-11 PROTEASES

Property	Protease 1	Protease 2	Protease 3
Adsorption to DEAE-cellulose	-	-	-
Adsorption to CM-cellulose (elution with M NaCl)	0	0.03	0.07
Anionic mobility in disc gels 8.6	-	-	-
Cationic mobility in disc gels pH 4.3 (cm. after 90 min.)	0.5	1.2	2.0
Collagenolytic activity	-	-	+
Sensitivity to Ionic Strength	+	+	+
pH optimum	9.3	9.5	8.5
EDTA sensitivity (Molarity)	4×10^{-6}	4×10^{-6}	4×10^{-3}
Molecular Weight	31,000	33,000	59,000

Table 9 show similarities between Protease 1 and 2, yet distinct differences between these and Protease 3. Are Proteases 1 and 2 distinct enzymes or are they merely the same enzyme altered by autodigestion? Is Protease 3 unrelated chemically or is it an aggregate, subunit, or precursor of the other two?

The above questions and possibilities for other avenues of research mentioned throughout this text indicate that Pseudomonas 461-3-11, initially isolated because of its action on curing steerhides, has far more potential than simply the investigation of collagenolytic activity.

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APPENDIX

A LIST OF SYNTHETIC MEDIA

In each of the following, metal compounds were added to 750 ml. of distilled water, the phosphate to 50 ml., the nitrogen sources to 100 ml., and the carbohydrates to 100 ml.. Each had their pH adjusted to 7.2 with NaOH and HCl. These were then autoclaved, combined and aseptically adjusted to a final pH of 7.2.

Medium A (by Eyzaguirre et al, 1973)

D-Glucose	10 gm.
NH_4Cl	6.6 gm.
K_2HPO_4	2.0 gm.
$FeSO_4$	13 mgm.
$MgSO_4 \cdot 7H_2O$	0.5 gm.
$MnSO_4 \cdot H_2O$	13 mgm.

Medium B

D-Glucose	9.0 gm.
NH_4Cl	4.06 gm.
K_2HPO_4	1.74 gm.
$FeSO_4$	11 mgm.
$MgSO_4 \cdot 7H_2O$	0.5 gm.
$MnSO_4 \cdot H_2O$	13 mgm.
$CaCl_2 \cdot 2H_2O$	11 mgm.

Medium C

D-Glucose	9.0 gm.
NH_4Cl	4.06 gm.
K_2HPO_4	1.74 gm.
$FeSO_4$	30 mgm.
$MgSO_4 \cdot 7H_2O$	0.28 gm.
$MnSO_4 \cdot H_2O$	17 mgm.
$CaCl_2 \cdot 2H_2O$	15 mgm.
$ZnCl_2$	14 mgm.
$CoCl_2 \cdot 6H_2O$	24 mgm.
$BaCl_2 \cdot 2H_2O$	24 mgm.
$SnCl_2 \cdot 2H_2O$	22 mgm.
$AlCl_3$	14 mgm.
$CuSO_4 \cdot 5H_2O$	25 mgm.
NaCl	0.58 gm.
$(NH_4)_2MoO_4$	0.10 gm.

Medium D

Glycine	7.5 gm.
NaH_2PO_4	1.38 gm.
D-Glucose	6.0 gm.
$FeSO_4$	15 mgm.
$MgSO_4 \cdot 7H_2O$	0.14 gm.
$MnSO_4 \cdot H_2O$	17 mgm.
$CaCl_2 \cdot 2H_2O$	15 mgm.
$ZnCl_2$	14 mgm.
$CoCl_2 \cdot 6H_2O$	24 mgm.
$CuSO_4 \cdot 5H_2O$	25 mgm.
$(NH_4)_2MoO_4$	20 mgm.

Medium E

NH_4Cl	5.3 gm.
NaH_2PO_4	1.38 gm.
D-Glucose	6.0 gm.
$FeSO_4$	15 mgm.
$MgSO_4 \cdot 7H_2O$	0.14 gm.
$MnSO_4 \cdot H_2O$	17 mgm.
$CaCl_2 \cdot 2H_2O$	15 mgm.
$ZnCl_2$	14 mgm.
$CoCl_2 \cdot 6H_2O$	24 mgm.
$CuSO_4 \cdot 5H_2O$	25 mgm.
$(NH_4)_2MoO_4$	20 mgm.

Medium F

KNO_3	10.1 gm.
NaH_2PO_4	1.38 gm.
D-Glucose	6.0 gm.
$FeSO_4$	15 mgm.
$MgSO_4 \cdot 7H_2O$	0.14 gm.
$MnSO_4 \cdot H_2O$	17 mgm.
$CaCl_2 \cdot 2H_2O$	15 mgm.
$ZnCl_2$	14 mgm.
$CoCl_2 \cdot 6H_2O$	24 mgm.
$CuSO_4 \cdot 5H_2O$	25 mgm.
$(NH_4)_2MoO_4$	20 mgm.

Medium G

Glycine	15 gm.
Cysteine	0.24 gm.
Methionine	0.34 gm.
NH_4Cl	1.06 gm.
D-Glucose	3.6 gm.
KH_2PO_4	2.72 gm.
NaCl	5.8 gm.

Medium H

Glycine	15 gm.
Cysteine	0.24 gm.
Methionine	0.30 gm.
NH_4Cl	1.06 gm.
Fructose	3.6 gm.
KH_2PO_4	2.72 gm.
NaCl	5.8 gm.